

THE EXPRESSION AND ROLE OF IL-4 AND IL-4(DELTΑ)2 IN TUBERCULOSIS WITH AND WITHOUT HIV CO-INFECTION

A thesis submitted by Dr. Keertan U J Dheda

MB.BcH (Wits), FCP (SA)

Centre for Infectious Diseases & International Health (Windeyer Institute of Medical Sciences) and Department of Thoracic and HIV Medicine (Royal Free Hospital NHS Trust), Royal Free & University College Medical School, London, UK

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ABSTRACT

Background: Tuberculosis progresses despite potent Th1 responses. A putative explanation is the presence of a subversive Th2 response. However interpretation is confounded by a novel cytokine, IL-4 δ 2, a splice variant and inhibitor of IL-4.

Methods: The expression of Th1 cytokines (IFN- γ), IL-4 δ 2, Th2 cytokines (IL-4) and sCD30 was investigated in whole blood, lung lavage and mononuclear cell cultures from donors with TB, TB-HIV co-infection, and matched controls.

Results: After validation of a fluorogenic real-time RT-PCR assay, the half-life of IL-4 mRNA, but not IL-4 δ 2, was found to be prolonged in TB vs controls ($P < 0.002$). mRNAs for IL-4 and IL-4 δ 2 were elevated in *unstimulated* cells from blood and lung lavage of patients vs controls ($p < 0.005$). Patients with TB expressed significantly greater mRNA levels of both cytokines in T-cells ($p < 0.05$ compared to controls where expression was predominantly in non-T cells). Radiological disease correlated with the IL-4/IFN- γ ratio and sCD30 ($p < 0.005$). Tuberculosis antigen upregulated expression of IL-4 relative to IL-4 δ 2 in mononuclear cell cultures from tuberculosis patients ($P < 0.05$). By contrast, though HIV-TB co-infected donors had increased IL-4 in blood and lung lavage, in lung the predominant form was IL-4 δ 2. After chemotherapy, in tuberculosis and in HIV-TB co-infection, IL-4 mRNA levels remained unchanged whilst IL-4 δ 2 increased ($p < 0.05$).

Conclusions: A Th2-like response, prominent in T cells, and driven by TB antigen, is present in TB and is modulated by treatment; suggesting a role for IL-4 and its antagonist, IL-4 δ 2 in the pathogenesis of TB and their ratio as a possible marker of disease activity. Furthermore, enhancement of IL-4 mRNA stability, a hitherto undescribed regulatory mechanism in human TB, may facilitate the immunopathological effect of IL-4. The specific antigens inducing the IL-4 response require identification to facilitate future vaccine development strategies. Further studies are required to determine whether IL-4 facilitates systemic HIV progression in co-infected patients.

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DEDICATION

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Publications relating to the work presented in this thesis (appended as supplementary material)

Original publications

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Reviews and correspondence

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ABBREVIATIONS

ActD (actinomycin D)	LAM (lipoarabinomannan)
Ag 85 (antigen 85)	LTBI (latent tuberculosis infection)
AMV (avian myeloblastosis virus)	MAPK (mitogen activated protein kinase)
APC (antigen presenting cell)	MDR-TB (multi-drug resistant tuberculosis)
ATP (adenosine triphosphate)	Mg ²⁺ (magnesium cation)
AU (adenylate-uridylate)	MgCl ₂ (magnesium chloride)
B2M (β2-Microglobulin)	Mn ²⁺ (manganese cation)
BA (β-Actin)	MMLV (Maloney murine leukaemia virus)
BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium)	MMP (matrix metalloproteinase)
CCR (chemokine receptor)	Mtb (Mycobacterium tuberculosis)
CD (Cluster of Differentiation)	MVS (<i>M. vaccae</i> sonicate)
CFP-10 (culture filtrate protein 10)	Nramp 1 (natural resistance associated macrophage protein)
CMI (cell mediated immunity)	OI (opportunistic infection)
CMV (cytomegalovirus)	PBMC (peripheral blood mononuclear cell)
CXR (chest x-ray)	PBS (phosphate buffered saline)
CYC (Cyclophylin)	PE (Phycoerythrin)
DC (dendritic cell)	PGK (Phosphoglycerokinase)
DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin)	PHA (phytohemagglutinin)
DTH (delayed type hypersensitivity)	PI3K (phosphatidylinositol-3-kinase)
dNTPs (deoxynucleotide triphosphates)	PMA (Phorbol Myristate Acetate)
EBV (Epstein Barr virus)	PPD (purified protein derivative)
ECM (extra cellular matrix)	PTB (pulmonary tuberculosis)
EF-1-α (Elongation Factor-1-alpha)	RCT (randomised control trial)
ERK (extracellular signal regulated kinase)	RFLP (restriction fragment length polymorphism)
ESAT-6 (early secreted antigenic target 6 kDa protein)	RPMI (Rosewall Park Memorial Institute)
FACS (fluorescence activated cell sorter)	RT-PCR (reverse transcription polymerase chain reaction)
FDA (Federal Drug Administration)	sIL-4R (soluble IL-4 receptor)
FITC (fluorescein)	SFC (spot forming cell)

GATA3 (GATA binding-protein 3)	SigH (sigma factor H)
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	Stat6 (signal transducer and activator of transcription 6)
GF (Growth Factor)	TB (tuberculosis)
GUS (β -Glucuronidase)	TBP (Transcription factor IID TATA binding protein)
HAART (highly active anti-retroviral therapy)	TfR (Transferrin receptor)
HIV (human immunodeficiency virus)	TGF (transforming growth factor)
HLA (human leucocyte antigen)	Th (T-helper)
HNP (human neutrophil peptide)	TLR (toll like receptor)
hsp65 (heat shock protein 65)	TNF (tumour necrosis factor)
HPRT (Hypoxanthine ribosyl transferase)	TNFR (TNF receptor-associated factor)
HuPO (Human acidic ribosomal protein)	TRAF (Tumor Necrosis Factor receptor-associated factor)
ICAM (intracellular adhesion molecule)	Treg (regulatory T cell)
ICCS (intra cellular cytokine staining)	TST (tuberculin skin test)
IGF I (insulin like growth factor 1)	UbcH5B (Ubiquitin conjugating enzyme)
IL- (interleukin)	VCAM-1 (vascular cell adhesion molecule 1)
IL-4 δ 2 (interleukin 4 delta 2)	whiB3 (transcriptional regulatory protein)
iNOS (inducible nitric oxide synthase)	whiB-like whiB3)
IPC (internal positive control)	
IRS (insulin receptor substrate)	

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1.1 Outline and objectives of the study

Tuberculosis (TB) is the commonest serious infectious disease worldwide and is responsible for ~ one fatality every 15 seconds! Despite an effective battery of drugs and the BCG vaccine, TB continues to flourish and ~ 40% of those with active disease fail to receive or complete treatment [1]. With the emergence of multi-drug resistant

TB, human immunodeficiency virus (HIV) and the failure of control and treatment programmes novel approaches to therapy are urgently needed. Therefore, some have argued that only a widely available vaccine offers any hope of controlling the worldwide pandemic. Although *M. tuberculosis* has been studied for over a century, surprisingly little is known about how a few organisms can evade, sometimes indefinitely, a highly sophisticated immune system.

Clinical experience indicates that, besides the virulence of the organism, host immunity plays an important role in the interaction with *M. tuberculosis*. Indeed, the majority of those exposed do not become infected [2-4], most people who become infected do not develop the disease [5] and many that develop tuberculosis have no obvious defect in host immunity [6]. In fact, Type 1 cytokines such as interferon- γ and IL-12, which are thought to be pivotal for successful host defence against *M. tuberculosis*, are prominent at the site of disease in those who do develop active TB [7, 8]. So why do these individuals contract the disease in the first place and why does it progress despite a vigorous type 1 response in the host? Moreover, why do some people appear to have natural resistance to the disease despite having a repeated high level of exposure [9]? Clearly, other factors that parallel IFN- γ production must be important for successful host defence. The aim of the current project is to shed further light on these crucial questions.

There are different T-lymphocyte and cytokine responses associated with the complex immuno-pathology of tuberculosis. Similarly, there are different hypotheses that may explain how *M. tuberculosis* may subvert protective immune responses (discussed later in this chapter) [10]. One tenable hypothesis (recently reviewed in detail in [11-13]), supported by increasing data, is that in susceptible individuals mycobacterial antigens invoke a small but significant IL-4 response that undermines the immune system thus allowing *M. tuberculosis* to evade cellular mycobactericidal host responses [14-16]. To clarify, this is not perceived to be a 'see-saw' like Th2 mediated downregulation of Th1 responses but an IL-4 mediated sabotage of protective macrophage responses within the framework of a dominant Th1 response. Although controversial, there is a growing body of evidence that an inappropriate Th2

(IL-4) response is present in those with TB [17-19], particularly in countries close to the equator [20-24].

Understanding this host response may lead to new immunotherapeutic approaches to prevention and treatment. Whilst it has been demonstrated that TB antigens drive the Th2/ IL-4 response in the peripheral blood cells of tuberculin skin test (TST) + healthy volunteers [25, 26], there are few and discordant data from donors with active tuberculosis [18, 21, 27-30]. If these findings are confirmed in donors with TB, it would be instructive to determine which specific antigens or epitopes drive IL-4. These epitopes/ antigens may be suitable for manipulation or deletion to facilitate the design of therapeutic or prophylactic vaccines, develop immunotherapeutic interventions that may have the potential to shorten current chemotherapeutic regimes or be an adjunct to the treatment of MDR- TB. Furthermore, defining whether (and which) TB antigens drive IL-4 would be important to develop strategies in reducing fibrosis, lung remodelling and necrosis [31], which is responsible for much of the morbidity associated with 'open pulmonary' tuberculosis. This has implications for prevention and spread of TB.

Previous work, though there are conflicting data [25, 27, 28, 30, 32], has suggested that expression of IL-4 is increased in tuberculosis compared to healthy controls. However, these studies do not take into account the presence of a natural antagonist and the only proven splice variant of IL-4, IL-4 δ 2 [33]. Moreover the discordant results are due to different culture conditions, cell types studied, antigen preparations used and a number of technical factors related to the measurement of IL-4, which is a low copy number cytokine and often beyond the detection limit of most assays [34] unless pre-stimulation protocols are used. The latter has its own set of problems [35]; the various technical aspects are discussed later. To circumvent the low sensitivity of immunoassays investigators, in mid-1990s, turned to reverse transcription polymerase chain reaction (RT-PCR), which amplifies reverse transcribed mRNA. However, this technique whilst good for qualitative purposes was notoriously unreliable for mRNA quantification [36]. There were also a number of additional technical drawbacks (discussed later).

More recently newer methods, using real time fluorogenic RT-PCR and microfluidic technology, have become available to measure levels of gene expression [37]. The new technology allows reliable and accurate quantification over a wide dynamic range [37]. It has, in the last few years, also become apparent that validation of reference genes used for normalisation (standardisation) of the raw PCR data is a prerequisite for accurate and meaningful study of gene expression [38] (the concept of normalisation is explained in detail in chapter 2, section 2.1). The use of unvalidated references may result in erroneous findings and misleading conclusions [39] (discussed in detail in chapter 2). Furthermore, there are now reliable and more precise methods to delineate latently infected contacts from non-infected healthy controls, another shortcoming of earlier studies [40, 41]. The above-mentioned methodological caveats and the inherent difficulty in reliably and accurately measuring IL-4 have, in part, been responsible for conflicting reports about the presence of a Th2 response in tuberculosis [17, 19, 27, 28]. Moreover, for practical reasons, three other factors confound the interpretation of existing data: (i) heavy reliance on *in vitro* and animal models, (ii) use of healthy or exposed subjects rather than human donors with the disease and (iii) reliance on data derived from whole blood rather than from samples acquired from the site of disease, the lung. Notably, previous studies have not studied IL-4 and IL-4 δ 2 within the context of HIV and HIV-TB co-infection. If IL-4 is fundamental to vaccine design then these studies may be crucial as HIV and TB are intertwined pandemics; in developing countries ~ 60% of TB cases are co-infected with HIV [1].

Accordingly, the objectives of the current project were:

(i) To first develop and validate a sensitive quantitative real-time PCR assay to overcome many of the drawbacks of earlier techniques attempting to measure IL-4 and IL-4 δ 2 in *unstimulated* cells (chapter 2 and 3). Once the assay was developed and validated, it was shown that use of conventional reference genes (like GAPDH) for data normalisation, as opposed to validated ones, might generate statistically different inter-group conclusions (chapter 2).

(ii) To gain further insight into the regulation of IL-4 the half-lives of IL-4 and IL-4 δ 2 mRNA were studied. Prolongation of IL-4 mRNA half-life has been shown in murine models of hypersensitivity pneumonitis [42] and the p38 signalling pathway may regulate the half lives of cytokines like IL-6 [43]. It was hypothesised that prolongation of the half-life of IL-4 mRNA was a putative regulatory mechanism modulating the biological effect of IL-4, possibly via the p38 signalling pathway. The results of these experiments indicated that in TB compared to control donors, the half-life of the mRNA encoding the agonist IL-4 was prolonged but not that of the antagonist, IL-4 δ 2, via a p38 independent pathway (chapter 4).

(iii) The next step was to establish whether, using the newly designed and validated assay, the gene expression of IL-4 and IL-4 δ 2 was elevated in biological samples from patients with active tuberculosis compared to healthy controls and to correlate these findings with clinical parameters (chapter 5). Moreover, a further objective was to establish whether Th2 cytokines were differentially expressed in the different body compartments (whole blood and lung lavage). Significantly, this was done immediately *ex vivo* after stabilisation of the sample to fix *in vivo* profiles. Furthermore, IL-4 and IL-4 δ 2 mRNA levels were investigated in relation to anti-TB treatment (chapter 5).

(iv) Having found that IL-4 and IL-4 δ 2 mRNA were up regulated in whole blood and lung lavage the next step was to determine the cell sub-populations making IL-4 and IL-4 δ 2 in TB and whether IL-4 δ 2 is expressed in CD8+ T cells of TB patients, as it is in systemic sclerosis [44]. These data revealed that, compared to healthy volunteers where most of the IL-4 was produced by non-T cells, the *unstimulated* T cells from TB subjects produced significantly more IL-4 mRNA than did cells from control subjects (chapter 6).

(v) To study the antigen-specificity of IL-4 and the biological relevance of these results, the ability of TB antigens to induce IL-4 and IL-4 δ 2 expression in peripheral blood lymphocytes from patients with tuberculosis were studied. These data indicated that in subjects with active disease TB antigen drives the expression of IL-4 rather than IL-4 δ 2. The results of these experiments are discussed in chapter 7.

(vi) The recent finding that IL-4/ IL-4 δ 2 ratio is significantly decreased in subjects with LTBI who remain healthy suggest that the ratio may be a marker of protection. Accordingly, we hypothesised that The IL-4/ IL-4 δ 2 ratio would be significantly increased in subjects with active tuberculosis (blood and alveolar lavage cells). The enhanced stability of IL-4 (but not IL-4 δ 2), the preferential induction of IL-4 (and not IL-4 δ 2) in antigen driven cultures and the significant change in IL-4/ IL-4 δ 2 ratio with anti-TB treatment support this contention. However, the observation that the IL-4/IL-4 δ 2 ratio remained unchanged in active tuberculosis, compared to healthy controls, may not be entirely compatible with this hypothesis. The implication of this finding is discussed further in chapter 5 and 9.

(vii) As the HIV and TB pandemics are inextricably intertwined IL-4 δ 2 expression, hitherto unstudied in HIV and HIV-TB co-infection, was also examined. These experiments revealed body compartment-specific differences between IL-4 and IL- δ 2 expression that may be important in the progression of HIV-TB co-infection (chapter 8).

(viii) In parallel soluble CD30 expression (a surrogate Th2 marker), not previously investigated in alveolar lavage or HIV-TB co-infection, was studied in TB and TB-HIV co-infection (chapters 5 and 8).

1.2. History, epidemiology and pathogenesis of tuberculosis

History and Origins of *Mycobacterium Tuberculosis*

Whole genome comparisons have revealed that the ‘modern’ TB strains are direct descendants of tubercle bacilli that existed before the *M. africanum* and *M. bovis* lineage separated from the *M. tuberculosis* lineage by progressive gene deletion [45]. The progenitor species may have been co-evolving with humans for ~ 3 million years [46]. Skeletons, dated to between 4000 and 1000 BC, suggestive of tuberculosis of the spine (Pott’s disease), have been found in Egypt, Jordon, Italy, Denmark and France

[47]. The oldest mummy with clear evidence of pulmonary tuberculosis dates back to between 1000 and 400 B.C. [48].

Although there is some data based on nucleic amplification techniques, there is little direct evidence to support the existence of pulmonary tuberculosis before 1000 B.C. Medical papyri from Egypt dating to 1600 B.C, which describe many chest diseases, make no reference to pulmonary tuberculosis. Similarly, ancient texts like the Old Testament or the Rig Vedas of the Hindus, written before 1000 B.C, do not refer to pulmonary tuberculosis. The earliest record comes from a clay tablet from the library of the Assyrian king, Assurbanipal (668 B.C.), which makes reference to haemoptysis, cough and night sweats. Inhabitants of the Indus river valley called the disease *jayashma* (wasting); the Greeks named it *ptthisis*. The more recent latin derivation in the English-speaking world was 'consumption'. These data suggest that pulmonary tuberculosis was a well-recognised clinical entity by the second half of the 1st millennium BC. It is worth noting that tuberculosis, while endemic in Europe since the early 1600's, was virtually unknown in Sub-Saharan Africa and the Indian subcontinent till the latter half of the 19th century [49].

To the ancient Hindus (people inhabiting the Indus river valley), tuberculosis was caused by stress in people with inherited susceptibility [47]; Hippocrates (5th century B.C) also suggested an inherited susceptibility. Francastoro in 1547 proposed the modern theory of contagion by microorganisms and in 1868 Villemon demonstrated that the cause of TB exists in an inoculable agent. It was only in that 1881 Koch isolated the tuberculosis bacterium [50]. It was another 60 years before Schatz and colleagues published their papers on streptomycin, which would lay the foundations for a reliable cure for tuberculosis.

Epidemiology

The WHO has estimated that tuberculosis causes 2.9 million deaths and 8 million new cases every year [1]. In developing countries, it causes one fifth of all adult deaths, one quarter of avoidable deaths and seven percent of all deaths. Remarkably, only ~ 60% of cases are cured and ~ 40% of cases remain untreated. After a continuous decline over 70 years there has also been an increase in the prevalence of tuberculosis

in the Western Europe and USA. This has been due to several factors including the emergence of HIV infection, cross-border migration, increasing drug abuse, socio-economic decline, delayed diagnosis and deteriorating public health infrastructure. Of particular concern is the emergence of MDR TB. Consequently, despite an effective array of drugs and the BCG vaccine, TB rates continue to burgeon worldwide. The WHO has now declared TB to be a 'global public health emergency' and there is an obvious and urgent need for more effective control measures.

The causative agent, *M. tuberculosis*, is transmitted by droplet spread. Variables that determine transmission of infection include cough, the presence of cavitation, smear positivity (suggesting at least 5 to 10 thousand organisms/ml of sputum), length of contact, intensity of exposure and the innate and CMI responses of the host. The two distinct phases of tuberculosis are those of infection and disease. In those with established infection viable bacilli lie dormant within macrophages and other cell types within the lung and elsewhere [51]. These individuals may be identified by means of a positive tuberculin reaction and more reliably by peripheral T-cell antigen-specific IFN- γ responses [40]. Generally, in high burden countries like the Gambia [52] and India [9] only about a third of high risk exposed subjects have positive antigen specific IFN- γ responses or TSTs [3]. If we accept these as sensitive measures of LTBI then it implies that most exposed subjects do not become infected. Furthermore, most people who become infected do not develop the disease (~90%).

However, a minority of infected individuals may develop progressive disease or reactivate their latent foci of tuberculosis at some stage in the future, when the balance between mycobacterial persistence and host cellular responses is disturbed. The estimated lifetime risk of developing reactivation disease in individuals not infected by HIV is approximately 10% (2 to 23%) [53]. It is noteworthy that infected persons may revert to a PPD negative status over time (5% per year) but it is unclear whether these persons eradicate persisting organisms [54]. Approximately 5% develop progressive primary disease, usually within 2-5 years of becoming infected [2]. Notably, a significant proportion has no obvious defect in host immunity such as malnutrition, chronic organ dysfunction or immuno-suppression.

The cause of active tuberculosis (re-infection from recent transmission vs re-activation of latent TB infection) in adults was controversial before the 1990s and was largely based on epidemiological modelling using historical tuberculin skin test data from the Netherlands [55]. The traditional view was that endogenous reactivation was responsible for 90% of adult tuberculosis [56]. More recently the use of molecular RFLP genotyping using IS6110 insertion sequence patterns, has revealed that 30% to 50% of adult tuberculosis is due to recent transmission [57, 58]. The prevalence of recent transmission was found to be approximately 40 to 50% in communities with a high prevalence of tuberculosis [59, 60]. In such communities 75% of cases of tuberculosis relapse are due to re-infection [61, 62]. In developed countries like the UK and Denmark 14% and 35% respectively, of tuberculosis cases was ascribed to recent transmission in a large studies (over 2000 isolates in each study) [63, 64]. By contrast, in the urban setting of New York City the prevalence of recent transmission was approximately 40% to 50% [58, 65]. Collectively these data suggest that in populations where the incidence of clinical tuberculosis is low reactivation predominates whilst, in populations with a high prevalence of tuberculosis or urban pockets in Western countries, recent transmission is increasingly important. This underscores the need to track and clinically follow-up close contacts of index cases. In summary, disease may develop because of progressive primary infection or later reactivation of LTBI, which in high burden settings constitute a minority of cases. The natural history of untreated infection is one of death within 5 years in 50%, self-cure in 30% and the remaining 20% persisting with chronic disease.

Pathogenesis

In a proportion of exposed individuals the infection progresses in the lung parenchyma and associated bronchopulmonary nodes (Ghon complex) [10]. Approximately 3 to 4 weeks later, the reason for this delay being unclear, T cells primed in the lymph nodes activate macrophages (also called CMI). The organisms either are destroyed completely or remain viable in latent foci. The fate of inhaled bacilli are summarised in figure 1.1.

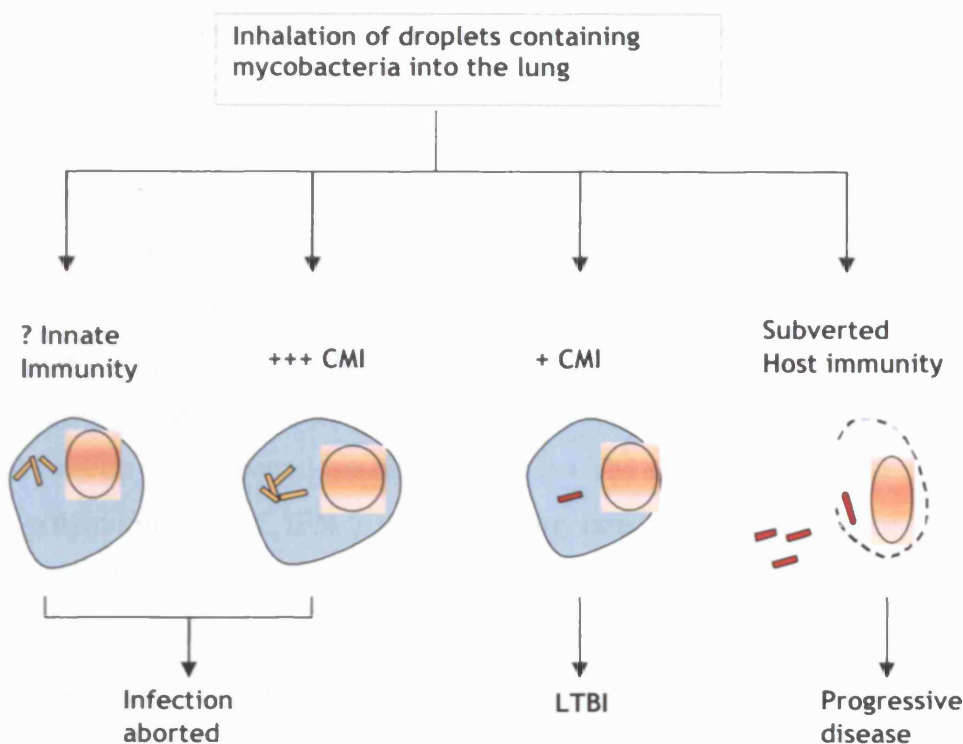


Figure 1.1. Fate of mycobacteria inhaled into the alveoli of the human lung. Presumably mycobacteria are destroyed by innate immune responses or CMI may be induced within the context of a granuloma. CMI may contain but not completely eradicate the organism (LTBI). In the minority progressive disease occurs. The factors that determine the fate of inhaled mycobacteria are poorly understood.

Host immunity plays an important role in the host-pathogen interaction in patients with TB; it is believed that when the balance between mycobacterial persistence and host cellular response is disturbed, latent infection may reactivate [10]. There are also genetic factors that determine initial infection rates and subsequent progression and these are likely to be important in apparently immunocompetent individuals. Nevertheless, what factors permit successful infection of the host remains unclear and the candidate genes that underpin these observations remain obscure. There is some evidence that genetic factors governing innate resistance to infection is important [66]. Indeed, some people appear to have natural resistance to the disease despite having a repeated high level of exposure [2, 3]. This is supported by data indicating that HIV impairs innate resistance thereby allowing much higher rates of tuberculous infection. Collectively, these data suggest that in most people exposed to *M. tuberculosis*, the organisms are destroyed either in the airways or presumably by the

early innate immune response in the lungs. Of the significant minority that become infected most contain the infection and do not develop active disease.

Nevertheless, in clinical practice in the UK most people who develop the disease outside the setting of HIV have no obvious defect in host immunity such as malignancy, starvation, diabetes mellitus or iatrogenic immunosuppression. So why do these people develop tuberculosis? One favoured notion is that there are inadequate bactericidal mechanisms due to a suboptimal Th1 response. However, most patients with tuberculosis have a vigorous Th1 response at the site of disease [7] and polymorphisms of IFN- γ and IL-12 are rare in clinical practice [67-69]. An alternative hypothesis implicates incapacitation of the macrophage mycobactericidal machinery; however, conclusive evidence from human studies is sparse and the immunological mechanisms facilitating these observations remain unclear. Another tenable hypothesis that may explain these observations is that, in susceptible individuals, certain conserved TB antigens corrupt the existing Th1 response by introducing in parallel a subversive Th2 response that results in progressive infection and immunopathology. Evidence for this hypothesis will be presented later (section 1.7.). What follows is a summary of our existing understanding of the immunology of tuberculosis infection and disease.

1.3. Diagnosis of latent TB infection

It is estimated that a third of the world's population is infected with *M. tuberculosis*. These individuals are asymptomatic and usually have normal chest radiographs. LTBI is classically diagnosed by performing a tuberculin skin test (TST) which measures CMI as a DTH hypersensitivity reaction to purified protein derivative (PPD); a precipitate of the protein fraction of a standardised batch of *M.tuberculosis* culture medium filtrate. When injected intradermally memory T cells that have encountered mycobacterial antigens in the past, not necessarily *M.tuberculosis* specific, will home in to the injection site initiating a DTH reaction. This is visible in 2 to 5 days as measurable local swelling and in some cases even ulceration. More recently peripheral blood derived T-cell IFN- γ responses to *M. tuberculosis* specific antigens [early secreted antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-

10)] have been investigated for the diagnosis of LTBI. The TST suffers from a number of problems which IFN- γ assays are designed to solve (table 1.1).

Furthermore, due to anergy, the TST is often unhelpful in individuals with chronic organ dysfunction, diabetes, malnourished or debilitated patients and those taking immunosuppressive treatments. Diagnosis of LTBI by the new test is based upon a vigorous T cell IFN- γ response, measured by ELISPOT assay or ELISA, to relatively *M. tuberculosis* specific antigens, ESAT-6 and CFP-10. Both the proteins are encoded on the region of difference (RD1) domain, a region associated with virulence and attenuation of BCG [70]. An ESAT-6 receptor has not been described and the precise function of these secreted proteins remains unclear (reviewed in [70]). Their expression is absent in BCG and most environmental mycobacteria except *M. marinum*, *M. szulgai* and *M. kansasii*; homologues of these proteins are also found in the genome of *M. leprae* [71].

There are two commercial assays available, which both measure overnight IFN- γ responses (< 24 hours) to overlapping ESAT-6 and CFP-10 peptides. The T SPOT TB assay (Oxford Immunotec, Oxford, England, see <http://www.oxfordimmunotec.com>) is an ELISPOT assay that uses peripheral blood mononuclear cells and has European CE Mark approval. QuantiFERON[®]-TB Gold (Cellestis, Victoria, Australia) is an ELISA utilizing whole blood and has both European CE Mark and American FDA approval (figure 1.2). The older QuantiFERON[®]-TB, which measured IFN- γ responses to PPD, is now superseded by QuantiFERON[®]-TB Gold, which is also available in a field friendly version, QuantiFERON[®]-TB Gold In-Tube, although there are no published data using the latter version (see <http://www.cellestis.com>).

Table 1.1. Comparison of factors impacting upon utility of the Tuberculin Skin Test and IFN- γ assay

Parameter	Tuberculin skin test	IFN- γ assay
1. Specificity	Cross-reactivity with BCG and environmental bacteria	Relatively M. tuberculosis specific
2. Workload	Requires return visit for which attendance is poor	Single visit
3. Chemoprophylaxis	Casts a wide net- may result in 'over treatment'	May avoid unnecessary treatment and toxicity
4. Subjectivity	Results dependent upon observer and technique and (1. above)	Provides 'yes'/ 'no' answer
5. Booster phenomenon	Yes	No
6. Cost	High in developed countries; low in developing countries	Affordable in developed countries
7. Longitudinal efficacy data	Plentiful	Limited (1 study)
8. Other factors	Prone to breakage of cold chain and syringe reuse in resource poor setting	Requires basic laboratory expertise

A

T SPOT TB assay

Purified PBMCs

Incubate in the presence of
M. tuberculosis antigens

Effector T-cells produce IFN- γ

IFN- γ binds to antibody on
the base of ELISPOT wells

Spots developed and
counted (manually or in
reader). Results as Spot
Forming Cells (SPC)

16-24 hours



Figure 1.2. The methods used for the two commercially available IFN- γ assays. In the T SPOT TB ELISPOT assay (A), PBMCs are separated from blood and incubated with antigen in plates of which the wells are precoated with an antibody that captures the IFN- γ .

For the QuantiFERON-TB Gold ELISA assay (B), the antigens are added to whole blood, and the supernatant is assayed for IFN- γ using a sensitive enzyme-linked immunosorbent assay.

Thus the former yields the total number of cells secreting IFN- γ , while the latter yields the total quantity of IFN- γ released. Numerous variants of these protocols are possible.

B

QuantiFERON®-TB Gold

Whole blood

Incubate in the presence of
M. tuberculosis antigens

Effector T-cells produce IFN- γ

Supernatant removed, and
IFN- γ measured by ELISA

Absorbance measured in
ELISA reader
Results as IFN- γ /ml

16-24 hours



The test relies on the principle that effector lymphocytes, which have very recently encountered antigen *in vivo*, will produce interferon- γ within hours when they encounter *M. tuberculosis* specific antigens. By contrast, memory T cells are more

likely to require several days in the presence of antigen before IFN- γ is produced [72, 73]. Therefore incubation of whole blood or PBMCs is for ~ 24 hours (16-20 hours for T-SPOT TB, 16 –24 hours for QuantiFERON[®]-TB Gold). This is to ensure that effector T cell responses are being measured. If longer incubation times are used it is possible for infection that has been treated or resolved to yield positive tests. This may explain the discordant results of studies that evaluated the change in IFN- γ responses with anti-TB treatment. [74-79]. Another crucial point is that the prevailing literature shows a marked heterogeneity in the type of assay used [enzyme linked (ELISA) versus immunospot (ELISPOT)], the type of antigen used (peptide versus protein), the number of antigens used (cocktails versus ESAT-6 and CFP-10 and PPD alone) and the type of test (in-house versus standardized protocol and reagents). These factors, particularly the latter, may account for inter-laboratory variability and is important to control for if valid comparison between investigators are to be made. Consequently, for practical and meaningful interpretation of tests that are available to the clinician or other investigators it is preferable to use a regulatory approved standardised assay that incorporate both ESAT-6 and CFP-10 antigens, and that use incubation times of < 24 hours. Hence, a standardised assay was chosen for the current study.

The lack of a gold standard for diagnosing LTBI was a problem that investigators faced when trying to determine performance outcomes of the IFN- γ assay. It is assumed that sensitivity should be at least as good as, or better, than in active tuberculosis, which tends to have lower IFN- γ levels due to the immunosuppressive effect of disease itself and trafficking of cells out of the peripheral blood compartment [78]. Based upon this arguable assumption the sensitivity was determined to be between 78 and 100% when both ESAT-6 and CFP-10 antigens were used in either an ELISPOT or ELISA overnight incubation assay [74, 80-83]. In a recent contact tracing study ELISPOT was found to be as sensitive as the TST for the diagnosis of LTBI [84]. Specificity was determined by studying T cell IFN- γ responses in study subjects who were asymptomatic and at low risk for LTBI. Specificity in these populations when both antigens were used, either in overnight ELISPOT or ELISA format, was 89 to 100% [80-83, 85]. Compared with the TST, the specificity of IFN- γ assay was considerably higher in BCG vaccinated individuals [83-85]. Although there

is no gold standard for LTBI, we know that the risk of LTBI is closely related to proximity and duration of exposure to infectious pulmonary tuberculosis cases. Investigators have therefore used precisely quantified TB exposure as a surrogate gold standard for LTBI [41, 85-87]. Ewer and colleagues, in one of the largest such studies, using an overnight ELISPOT assay incorporating both antigens and performed 8 weeks post-exposure in a school tuberculosis outbreak, found that ELISPOT correlated significantly better with duration and proximity of TB exposure than did TST, suggesting increased sensitivity with ELISPOT [41]. Furthermore ELISPOT but not TST was independent of BCG vaccination status indicating higher specificity than TST. The authors suggest that the higher specificity of the IFN- γ assay allows for more precise targeting of anti-TB treatment in low prevalence countries. Concordance between the TST and IFN- γ assay was 89% [41] and similar in other studies using overnight incubation with both antigens (74 to 94%) [9, 52, 81]. There is a paucity of data on assay reproducibility, changes in responses over time and with treatment of LTBI, and how often indeterminate results occur (failure of the test's internal positive control). However, a recent study reported that in an unselected hospital population being evaluated for LTBI using a standardised ELISA assay, ~ 25% (mainly subjects with some form of immunosuppression) had indeterminate reactions [88]. In another immunosuppressed (HIV+) but smaller cohort, only 3% of participants were found to have indeterminate reactions when using an ELISPOT assay [89].

IFN- γ assays have also been investigated in other mycobacterial infections: positive IFN- γ responses to either ESAT-6 or CFP-10 antigens are found in patients with clinically active *M. marinum* and *M. kansasii* disease [83, 90], individuals with high environmental mycobacterial exposure such as flower sellers and tropical fish tank owners [90] and Brazilian subjects with leprosy [91-93]. It has been shown in low burden countries that a significant proportion of positive TSTs are due to *M. avium* infection [94, 95] but the IFN- γ assays remain negative in the face of proven *M. avium* disease [96]. Consequently, the standardised IFN- γ assays will need validation in populations who come from or reside in countries where both tuberculosis and leprosy are endemic, and where there is a high environmental mycobacterial load [97].

Collectively these data indicate that, when a combination of antigens are used (ESAT-6 and CFP-10) in either overnight ELISA or ELISPOT format, IFN- γ assays have a

high sensitivity for diagnosing presumed LTBI and agreement between the TST and IFN- γ assays are good. Nevertheless, in the absence of a gold standard the absolute sensitivity of the IFN- γ assay, and the TST for that matter, for diagnosing LTBI remains unclear. However, IFN- γ assays when compared with the TST have greater specificity in BCG vaccinated subjects and in the case of T-SPOT TB display a stronger association with TB exposure. IFN- γ assays are less biased by environmental mycobacteria like *M. avium*, which is responsible for significant number of false positive TST reactions [95]. This specificity will facilitate more accurate targeting of treatment for LTBI. To what extent a heavy burden of environmental mycobacteria and *M. leprae* influence the performance of IFN- γ assays requires further study [97]. Despite the shortcomings of the TST, large studies have shown that treatment of LTBI, as defined by the TST, substantially reduces the risk of developing active disease [2, 98, 99]. Consequently, the important clinical question is whether treatment of those identified as having LTBI by the IFN- γ assay will actually reduce the incidence of subsequent clinical tuberculosis (TB). Secondly, relevant to both industrialized and developing nations, it remains to be determined whether IFN- γ responses are predictive for high risk of progression to active tuberculosis. Long term and appropriately powered prospective studies are required, and currently underway, to answer these questions.

In summary, the antigen specific IFN- γ assay has revolutionised the diagnosis of LTBI in low prevalence countries. It retains specificity in those who are BCG vaccinated or have a false positive TST due to environmental mycobacteria. Two standardised commercially available tests will make future comparisons between studies considerably more meaningful. The implications for research into host immunity are significant: sharpening of the blurred TST based distinction between infected and uninfected exposed contacts will facilitate the investigation of correlates of protective host immunity.

1.4. Immunology of tuberculosis

There is a large interplay of different cell types and cytokines in tuberculosis infection and immunity. In most people an intricately orchestrated cellular immune cascade successfully eradicates the organisms; even in infected individuals the immune system is able to maintain the pathogen in a state of latency in the majority. However, it is still unclear what specific mechanisms lead to successful host defense and what precisely constitutes correlates of protective immunity.

Whilst being invaluable to our current understanding of tuberculosis related immunology prevailing data are complicated by several factors. For example some of the immunological pathways pertaining to mice, in which cavitation (a major determinant of transmission) does not occur, may not be applicable to humans. The ability of murine macrophages [100] but not human ones [101] to cause bacteriostasis of *M. tuberculosis* is a case in point. Moreover, human leucocytes responding to heat killed antigen preparations of *M.tuberculosis* may respond very poorly to live bacteria [102]. Lymphocyte responses and mycobacterial antigen profiles may vary according to bacterial strains, culture conditions [103] and the viability organisms when live mycobacteria are used. Furthermore, *in vitro* studies in artificial laboratory micro-environments are non-physiological and such data may not necessarily be extrapolated to the *in vivo* human model. Finally, immunological pathways delineated in human whole blood may not reflect the conditions at the site of disease (lung or lymph nodes). Nevertheless, the ever-increasing accumulation of scientific knowledge is likely to elucidate new immunological pathways and molecules critical for host defence. These findings will likely facilitate new immunotherapeutic interventions to manage TB. This may involve the development of prophylactic or therapeutic vaccines, administration of pro-inflammatory cytokines such as aerosolised IFN- γ used in treatment resistant MDR-TB [104], neutralising antibodies to immunosuppressive cytokines or inhibitors of molecules like TNF- α that may cause immunopathology e.g. thalidomide used to treat TNF mediated granulomatous diseases like erythema nodosum leprosum and sarcoidosis.

There are different T-lymphocyte responses, cell sub-types and cytokines associated with the complex immuno-pathology of tuberculosis. They will be discussed within the framework presented in table 1.2.

Table 1.2. Cytokines and cell types implicated in host defense against *M.tuberculosis*

Type 1 cytokines (IFN- γ , IL-12, IL-23)
IL-4 δ 2
TNF- α
Type 2 cytokines (IL-4, IL-13)
Other cytokines and chemokines
Macrophages, Dendritic cells
CD4+ T cells, CD8+ T cells, $\gamma\delta$ T cells, CD1 restricted T cells, regulatory T cells
Other cell types (fibroblasts, epithelial cells, granulocytes)

1.4.1. Effective granuloma formation, Macrophages & Th1 cytokines

The different clinico-pathological phases of tuberculosis and their immunological equivalents may be viewed, as ‘a series of battles between host and invader’ [105]. The first cell types encountered by inhaled mycobacteria are the alveolar macrophages; phagocytosis is mediated by various host receptors including toll-like-receptors, macrophage mannose receptor, CD 14, complement receptor 3, Fc receptors and cholesterol [106-108]. Other cell types like epithelial cells, neutrophils, endothelial cells and fibroblasts may also be infected with *M. tuberculosis* [51] and may serve as reservoirs for bacterial multiplication.

Lung $\gamma\delta$ T cells, NK T cells and granulocytes are important early arrivals that precede the second wave of expanded, IFN- γ and TNF- α producing, effector T cell populations [109-111]. Collectively these cells and molecules initiate cross talk

between the innate (antigen presenting cells, TLRs, defensins, surfactant protein etc) and adaptive immune systems (primed T cells). Consequently a chemokine and cytokine cascade which, attracts other macrophages and later T cells to the site of infection, is initiated [112] (figure 1.3).

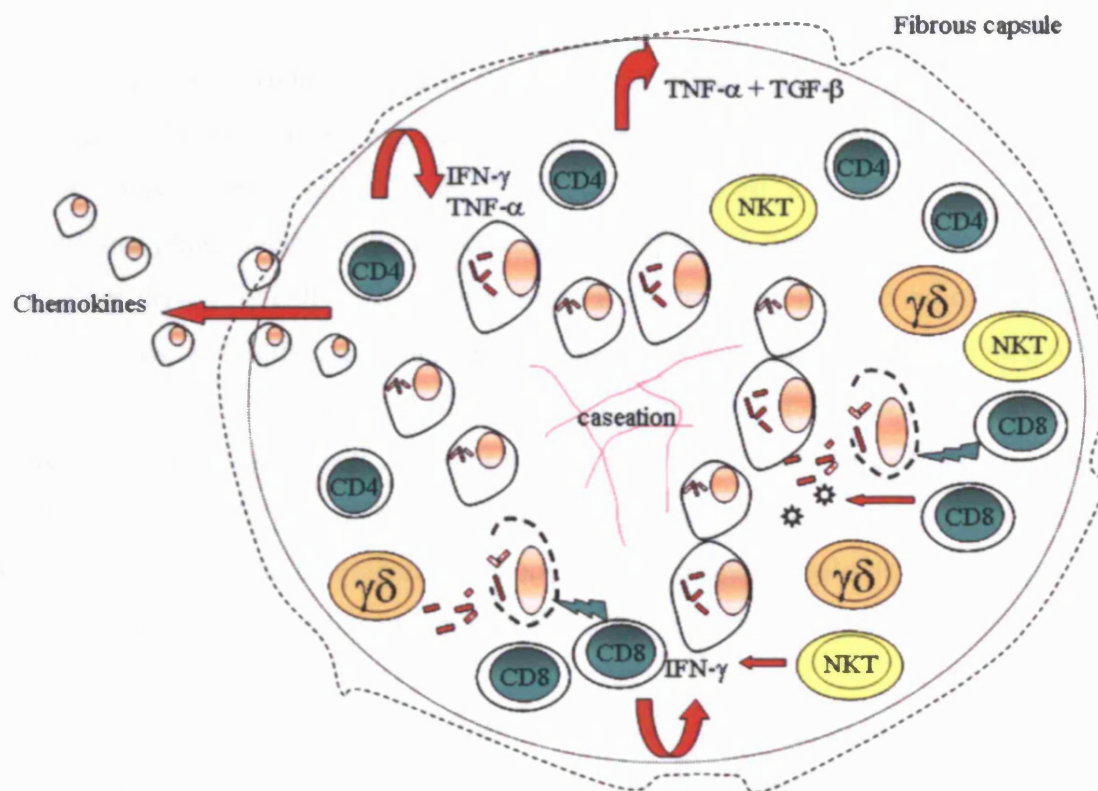


Figure 1.3. Principle cell types and cytokines involved in competent granuloma formation. NK T cells (NKT), CD4+ T cells, CD8+ T and $\gamma\delta$ T cells produce Type 1 cytokines that activate macrophages, which are mycobactericidal. Granulocytes (not shown) may be important in early granuloma formation. Mycobacteria (rods) may be present within cells or extracellularly. Mycobacterial killing can occur by macrophage apoptosis, cytotoxic T-cell lysis (jagged arrow) or directly through granulysin (star) mediated destruction. TNF- α is a likely requisite for macrophage mycobactericidal activity, necrosis and formation of the fibrous capsule. Cellular recruitment into the granuloma is facilitated by a chemokine gradient.

Antigen presenting cells (macrophages and dendritic cells) produce IL-12; polarising cells to a Th1 type of response. IL-12 is a requisite for *M. tuberculosis* induced murine T cell proliferation [113], is crucial for IFN- γ induction [114] and mutations

of genes for IL-12 or its receptor have been associated with mycobacterial disease in humans [114, 115]. The activated and expanding population of T cells produce IFN- γ , which activate mycobacterium-laden macrophages (also called CMI or acquired cellular resistance) [101, 116]. 1,25-dihydroxy vitamin D3 may be important for this activation in humans [117] possibly by activation of anti-bactericidal peptides.

There is plasma exudation and a fibrin clot is formed [118, 119]. Macrophage aggregation to form an early granuloma core is mediated by hyaluronic acid [120], which binds to macrophages via CD44 [121]. Detailed histological analyses of developing granulomata in the rabbit model [122] led to the view that macrophage activation driven by cell-mediated immunity is followed by destruction of the same macrophages, which then accumulate as caseous necrosis and is often associated with death of the contained bacilli. An alternative view is that granulocytes are early arrivals that mediate the cell lysis that forms the core of developing caseous necrosis [111, 123, 124]. Recent pathological studies of granulomas in tuberculous human lungs indicate that the peripheral lymphocyte zones of the granuloma have secondary lymphoid follicles analogous to those found in lymph nodes [111].

Macrophages and other cell types like fibroblasts, endothelial cells and neutrophils also produce proteases [metalloproteinases (collagenase, gelatinase and stromelysin); the lysosomal proteinases (cathepsins); and the plasminogen/ plasmin system and its activator urokinase]. Such enzymes may facilitate granuloma formation by mediating the antigen processing, removal of ECM and cellular debris, and the processing of cytokines and hormones [125]. They are tightly regulated at multiple levels including transcription, proenzyme formation, signalling control, and by tissue inhibitors of metalloproteinases (TIMP) [126, 127].

Within the granuloma both T cells and macrophages secrete TNF- α and lymphotoxin α_3 . Not only is TNF- α crucial for murine host defence [128, 129], but it also facilitates the structural integrity of the granuloma by mediating the formation of the encapsulating fibrous wall [130] together with TGF- β [131, 132] (figure 1.4). Macrophages also secrete insulin-like-growth-factor-1 (IGF-1), fibroblast-GF, fibronectin and platelet-derived-GF [108, 133]. These growth factors are chemotactic

and support macrophage proliferation and hence laying down of ECM comprising collagen, fibronectins and glycosaminoglycans [134, 135]. The granuloma develops a caseous centre surrounded by a concentric zone of inflammatory cells encircled by collagen and newly laid extracellular matrix. Granulomas are present in varying stages of development and the granulomas may contain mycobacterium-laden macrophages in diverse stages of maturity. Caseous necrosis, which occurs at about 2 to 3 weeks in the rabbit model [122], and its associated low oxygen content create unfavourable conditions for mycobacterial multiplication [136]. This together with macrophage activation and CD3+ effector mechanisms culminates in mycobacterial sterilisation. It was shown in 1927 that healed primary lesions are usually sterile within 5 years, though latent bacilli may persist in other, superficially normal, parts of the human lung [51, 137].

Controlled dissolution of the granuloma follows mycobacterial containment, though this may be incomplete if there has been massive production of caseum. Proteases cleave components of the ECM. Macrophages phagocytose the partly degraded ECM components, which are terminally degraded within the lysosome or transported out of the lung [138, 139]. Minor residual scarring may remain.

1.4.2. Dysregulated granuloma turnover and liquefactive necrosis

In a significant minority, those with progressive primary and reactivation disease, macrophage and other innate mycobactericidal mechanisms fail despite high levels of IFN- γ . The putative mechanisms involved and the potential role for IL-4 are discussed below (section 1.5 and 1.6). Instead of co-ordinated mycobactericidal granuloma formation there is liquefactive necrosis. This forms an ideal culture medium for mycobacteria; they multiply extracellularly to large numbers [105] as macrophages are unable to survive in necrotic tissue, partly because of its toxic fatty acid content [136]. The proteolytic enzymes [140] compromise the integrity of the fibrous granuloma capsule. Caseous material may discharge into surrounding blood vessels and airways with dissemination systemically and to the outside environment via cough induced respiratory droplets. The cavity often has an external zone of collagen internal to which is a zone of granulation tissue rich in fibroblasts, inflammatory cells and capillaries [105].

Although the exact mechanisms of liquefaction are unknown, the available data suggest that dysregulated proteolysis and release of cellular lysosomal contents [140-142], direct mycobacterial toxicity (virulence factors like polyketide toxins and ESAT-6), the Koch [143] and Shwartzman phenomena [144], and host effector cells and cytokines are key players (figure 1.4). Granulocytes may be important facilitators of early necrosis [111, 123] in murine and human models. As discussed below, local tissue damage will be more likely if the macrophages undergo necrosis rather than apoptosis. Furthermore, certain deletional mutants of *M. tuberculosis* have unchanged ability to proliferate within the host, but induce much less immunopathology suggesting that mycobacterial virulence factors actively induce tissue damaging CMI responses [145, 146].

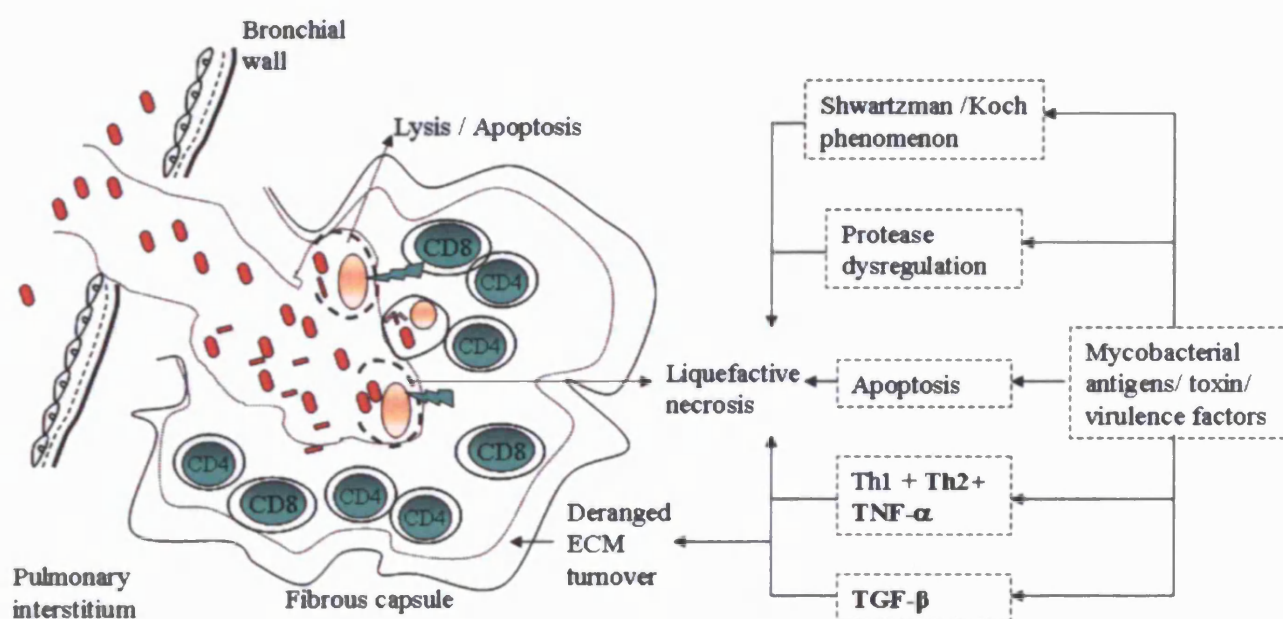


Figure 1.4. Factors that may contribute to dysregulated granuloma formation. Mycobacterial antigens mediate uncoupled protease metabolism activity, apoptosis, the Koch phenomenon, and perhaps cell lysis mediated by ESAT-6, and induce cytokine release (TNF- α , mixed Th1/Th2 and TGF- β), which collectively facilitate liquefactive necrosis and deranged extracellular matrix (ECM) turnover. Mycobacteria (solid capsules) multiply extracellularly, compromise the fibrous granuloma capsule and may discharge into a bronchial lumen or blood vessel.

1.4.3. The Koch phenomenon

Koch showed that tuberculous guinea pigs developed necrosis locally and at distant sites of infection upon re-challenge with tuberculin and high tuberculin reactivity occurs in experimental animals with liquefaction [147]. Similarly the tuberculin test is frequently necrotic in humans with tuberculosis but not in healthy BCG vaccinated individuals [105]. More recently, an elegant study showed that *M. tuberculosis*-infected mice developed increased lung inflammation and elevated TNF- α levels when rechallenged with mycobacterial antigens [148]. Moreover, when prophylactic vaccine candidates are tested for therapeutic effects in mice with tuberculosis they develop increased immunopathology [149] and classical Koch reactions characterised by cellular necrosis within granulomata [150]. One explanation may be the systemic Shwartzman reaction, which referred originally to cutaneous necrosis at a site of a previous endotoxin injection after intravenous injection of LPS [151]. Indeed, *M. tuberculosis*-infected C57BL/6 mice, which do not conventionally develop necrosis in this model, develop caseous necrosis after inoculation with LPS [152].

TNF- α

There are several lines of evidence to suggest that TNF- α is crucial for host defence against tuberculosis and containment of latent infection: TNF- α together with IFN- γ activates murine macrophages [116], facilitates encapsulation of the granuloma [130] and monoclonal antibodies to TNF- α cause reactivation of latent infection in mice and humans [129, 153].

Although TNF- α is essential for immunity to tuberculosis, in progressive human disease TNF- α is associated with fever and wasting and correlates with disease activity and immunopathology [154-157]. Human cells containing *M. tuberculosis* are rendered exquisitely sensitive to killing by TNF- α [155, 158]. As already outlined, TNF- α can up regulate metalloproteinases and urokinase and thereby facilitate proteolysis of structural lung elements. However, under what conditions is the protective TNF- α molecule toxic? Data, as outlined in section 1.6 below, support the

synergistic role of Th2 cytokines in such toxicity and as a crucial facilitator of the Koch phenomenon, an entity that has never satisfactorily been explained immunologically.

1.4.4. Other cell types and cytokines relevant to TB immunology:

Dendritic cells

Dendritic cells (DC), the sentinels of lung immunity, are abundant in the airway, alveoli and lung parenchyma [159]; they possess pattern recognition receptors including TLRs and are able to internalise *M.tuberculosis* [160]. DC-specific intracellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) is one of many adhesion molecules that can bind *M. tuberculosis* [161, 162] and whose expression is upregulated by IL-4 [163] and IL-13 [164]. The early innate immune response includes interaction with $\gamma\delta$ and CD1 restricted T cells, which produce macrophage activating effector cytokines. Antigen presentation may occur within the context of MHC class II molecules, group 1 CD1 molecules (glycolipid antigens) [165] and antigens from apoptotic cellular fragments may be presented by MHC class I molecules via the exogenous pathway [166]. Stimuli like TNF- α result in maturation of DCs to a phenotype that is capable of antigen presentation and T cell stimulation [167]. DCs then home in to the lymphoid organs where T cell growth and differentiation of antigen specific T cells occurs.

Significantly, unlike other APCs DCs can initiate and direct an immune response. Antigen can polarise T cell responses toward Th1 in the presence of DC derived IL-12 [168-170] and IFN- γ [171] or Th2 in the presence of IL-4 [172]. The type of organism and antigen specificity plays a role: different antigenic components of the same microbe may cause Th1 or Th2 polarisation by murine DCs [173]. BCG induces IL-4 production by DCs generated from infected monocytes suggesting that mycobacterial antigens can induce DC mediated Th2 polarisation [174]. Further evidence that dendritic cells may direct T-cell differentiation comes from evidence that certain DC subsets (CD11c+ CD14-) may polarise T cells to a Th2 phenotype [175]. Recently, evidence has emerged that APC use the Notch pathway to instruct T cell

differentiation. Notch directs Th2 differentiation by inducing GATA3 and by directly regulating IL-4 gene transcription [176]. Finally DCs may also play a role in inducing peripheral tolerance to self antigens and driving Treg [177]; it remains to be determined if this function interacts with host resistance to mycobacteria.

CD4+ and CD8+ T cells

CD4+ T cells are essential for host defense against mycobacteria. Both mice and humans deficient in CD4+ T cells rapidly reactivate dormant *M.tuberculosis* organisms; HIV+ subjects with CD4+ lymphopenia are exquisitely susceptible to TB infection [178, 179]. Human T cells are crucial for IFN- γ dependent macrophage activation and may also have direct mycobactericidal effects via a cytotoxic T cell response [180]. There are relatively few data on the cytokine profiles generated by PBMCs from TB patients. *In vitro* studies have produced conflicting results about cytokine production by CD4+ T cells with some studies reporting predominance of Th1 cytokines while others report a mixed Th1 and Th2 like response in peripheral blood (the discordant data are discussed in detail in section 1.7.2.). Nevertheless, these data have a number of limitations: they must be interpreted within the context of translocation of antigen specific CD4+ T cells to the lung, the data may not be meaningful without measuring the Th2 antagonist IL-4 δ 2 and some techniques like in situ hybridisation are relatively insensitive for IL-4 detection.

Murine CD8+ T cells are instrumental to successful host immunity against tuberculosis [181, 182]. There are 3 effector functions of CD8+ cells: production of type 1 cytokines that activate macrophages, lysis of *M.tuberculosis* laden macrophages and direct killing of mycobacteria. The latter is mediated by perforin and a granule associated protein, granulolysin [183]. There are no studies addressing the question of IL-4 δ 2 production in CD4 and CD8+ T cells in tuberculosis.

$\gamma\delta$ and CD1 restricted T cells

$\gamma\delta$ T cells are thought to be important in the early phase of the immune response to murine tuberculosis [110, 184]. Activation is followed by release of type 1 cytokines

and granulysin-mediated lysis of macrophages and dendritic cells [185]. In addition to TNF- α and type 1 cytokines, $\gamma\delta$ T cells may also produce IL-4 and IL-10 [186].

In contrast to the MHC family of proteins, which present peptide antigens, the CD1 family of proteins serve as an antigen-presenting pathway for glyco-lipid based antigens. Structurally the CD1 molecule is a heterodimer and is widely distributed on haemopoietic cells including thymocytes, monocytes (but not macrophages), dendritic cells and B cells [187]. A large part of the T cell population (CD4-CD8-; CD4+; CD8+ T cells) can recognise CD1 restricted antigens [188]. Mice deficient in group 2 proteins (CD1d) do not show increased susceptibility to TB [189]. Nevertheless, there is a higher frequency of CD1 restricted T cells in those with tuberculosis compared to controls [190]. CD1 restricted T cells produce macrophage activating type 1 cytokines [110] and also display granulysin mediated cytotoxic lymphocyte activity [191]. These effector functions may contribute to early innate mycobacterial killing. NKT cells may also produce IL-4 in the early phase of infection. It is intriguing to speculate that in susceptible individuals, as demonstrated in a encephalomyelitis model [192], mycobacterial antigens drive NKT cell mediated IL-4 production, which facilitates the subversion of mycobactericidal innate immune responses.

Transforming growth factor β

Various cell types including activated macrophages, fibroblasts, dendritic cells, neutrophils and activated lymphocytes produce and are sensitive to the effects of TGF- β , which exists as 3 isoforms (β 1, β 2 and β 3), binds to one of two receptors (type I and II), and signals through transmembrane serine/threonine kinases and transcription factors (SMAD proteins) [193]. Levels of the mature protein are regulated by protease (e.g. plasmin) mediated cleavage of latency-associated peptide (LAP) from the bioactive dimer [193].

In immunocompetent individuals TGF- β enhances monocyte chemotaxis, augments the expression of Fc receptors and takes part in the reversible fibrous encapsulation of the granuloma [131, 132]. On the other hand it has anti-inflammatory properties that modulate adhesion, apoptosis, proliferation and differentiation of various cell types

including lymphocyte proliferation and IFN- γ induced macrophage activation [194]. TGF- β is induced by mycobacterial antigens [195] and TGF- β expression is increased in PBMCs, monocytes and granulomas from patients with tuberculosis [196]. Data suggest that TGF- β enhances intracellular growth of *M.tuberculosis* in humans [197] and inhibits the antimycobacterial effects of TNF- α [198]. The putative role of TGF- β and IL-10 in suppressing protective host responses is discussed in section 1.5 and the putative role of IL-4 in facilitating TGF- β mediated immunopathology is discussed in section 1.7.5.

Other cytokines and chemokines

Studies in KO mice have highlighted the importance of pro-inflammatory cytokines like IL-1 [199] and transcription factors (CCAAT/enhancer binding protein and NF- κ B), which are common pathways for the expression of multiple cytokine genes including IL-1 β , IL-6 and TNF- α [200] for efficient mycobacterial control. However KO models of IL-6, a B-cell growth and differentiation factor, suggest that IL-6 is not essential for protective immunity [201]. IL-8 is chemotactic for neutrophils, lymphocytes and basophils; IL-8 is elevated in tuberculosis, correlates with TB disease severity [202] and is associated with tissue necrosis in TB (discussed in section 1.7.5.1).

Data on the role of IL-13 in tuberculosis are sparse. IL-13 has similar properties to IL-4, namely, downregulation of Th1 responses and inflammatory cytokines, and IgE class switching. IL-13 and IL-4 bind to receptors that share the IL-4 receptor α chain. IL-13 is upregulated in exposed contacts [203] and those with active tuberculosis and correlates with IL-4 levels [17].

Chemokines are cytokines that recruit inflammatory cells to a site of infection; adhesion molecules facilitate this action. Expression of a variety of chemokines and their receptors (RANTES, MCP-1, fractalkine, MIP-1 α , MIP-1 β) are up regulated in various cell types and in the different body compartments of patients with tuberculosis [204]. Collectively, these data imply an intricate system of signalling between different compartments that coordinate effective granuloma formation and a

competent host immune response.

1.4.5. Apoptosis in tuberculosis

Several reports documenting apoptosis of *M.tuberculosis* infected human macrophages *in vitro* and *ex vivo*, indicate that only live bacilli, rather than heat killed components of *M.tuberculosis*, can initiate apoptosis [205-207], suggesting that a secreted product of bacterial metabolism may be responsible. Apoptosis of infected macrophages is facilitated by TNF- α though other mechanisms (Fas-FasL interaction, purinergic receptors, ATP, H₂O₂) have also been shown to activate pathways of cell death.

Apoptosis may be beneficial to the host. Both in mice [208] and in man [209] CD8+ cytotoxic T cells that use the granule-mediated pathway to induce apoptosis (as opposed to the Fas-FasL pathway) lead to killing of the contained mycobacteria [210]. Apoptosis induced by ATP [211, 212], TNF- α independent of IFN- γ [129], Fas Ligand [213] and hydrogen peroxide [214] all promote killing of virulent *M. tuberculosis* within macrophages, whereas the necrotic mode of death does not [213, 215]. Moreover, several reports suggest that apoptosis reduces bacterial viability compared to H₂O₂ or complement mediated killing [213]. Apoptotic blebs may facilitate antigen presentation by dendritic cells [166] and phagocytosis of apoptotic vesicles may enhance bacterial killing compared to sonicate induced necrosis of macrophages [216].

By contrast other reports suggest that apoptosis is deleterious to the host. Reduced apoptosis of lymphocytes has been shown to correlate with improvement on anti-TB treatment [217] and reduced burden of *M.avium* infection in mice [218]. Consequently, it has been suggested that apoptosis may play a role in weakening protective responses and contributing to immunopathology by deletion of protective T cell clones or alternatively lack of deletion of immunosuppressive clones [219]. Apoptosis may also facilitate immunopathology. Indeed, large numbers of apoptotic T cells and macrophages are seen in caseous foci [220], which unlike necrotic cells, are usually removed with minimal inflammation. Furthermore, it is also possible that

when a sufficient percentage of the cells are undergoing apoptosis, the normal clearance mechanism fails, and apoptotic cells can themselves contribute to caseum. IL-4 may facilitate this process by sensitising *M.tuberculosis* reactive lymphocytes to TNF mediated apoptosis [26] (discussed in greater detail in section 1.5).

1.4.6. Genetic influence on susceptibility and resistance to tuberculosis

The remarkable decline in tuberculosis death rates of American Indians of the Qu'appelle valley, who had no longstanding historical exposure to TB, indicate that natural selection drives the differential survival of phenotypes [221]. Furthermore, the increased susceptibility of Afro-American subjects to tuberculosis despite equivalent exposure [222], studies in twins [223], and the differential disease outcome and susceptibility after accidental immunisation of 251 German infants with a virulent strain of *M.tuberculosis* [221], all suggest that genetic factors play a role in host susceptibility. The failure to convert to tuberculin sensitivity in many individuals, despite repeated high-level exposure, supports this contention [9]. Nevertheless, the genes that govern susceptibility remain obscure.

MHC genes that have been found to be associated with disease susceptibility include HLA DR2 in different populations and the DQB1*0503 allele in Cambodians [66]. Non-HLA gene polymorphisms include those of IFN- γ and its receptor, IL-12 receptor, the IL-1 gene complex, mannose binding protein and the vitamin D receptor [66]. Data suggest that genetic factors govern the ability of host innate immune responses to prevent infection. Nramp 1 (natural resistance associated macrophage protein) is expressed in the endosomal/ lysosomal compartment of mouse macrophages and serves as a membrane cation pump [224]. Human Nramp 1 shares 88% of the mouse nucleotide sequence, encodes a membrane transport protein and is strongly expressed in lung neutrophils. Although Nramp 1 deficient mice do not display greater susceptibility to TB [225] studies in human tuberculosis have shown Nramp 1 polymorphisms to be associated with susceptibility in Gambians but not in Cambodians [226, 227]. Similarly, IFN- γ gene polymorphisms are no commoner in Gambians with TB compared to healthy control donors [68]. These findings indicate that associations between tuberculosis and polymorphisms are strongly influenced by

ethnicity. Notably, there are few data on IL-4 gene polymorphisms and their relationship to disease susceptibility. Data from a Peruvian group published in abstract form suggest that an IL-4 gene polymorphism may modulate the risk for developing disseminated forms of tuberculosis [228]. However, details remain unpublished and it remains unclear whether this represents a loss or gain of function mutation; attempts to contact the authors have been unsuccessful.

1.4.7. Th1 / Th2 paradigm

There are 2 overlapping subgroups of CD4⁺ T cells that differ in the production of their cytokine profile i.e. the Th1 and Th2 groups of cells [229, 230]. The Th1 cells produce IL-2 and interferon gamma (IFN- γ) and are crucial for delayed hypersensitivity and tend not to produce IL-4. Th2 cells on the other hand produce IL-4, IL-5, IL-6 and IL-13 and are important for B cell, eosinophil and mast cell growth and differentiation. In humans, unlike mice, other cytokines like IL-10 and TNF- α are produced by both groups of cells. Both Th1 and Th2 groups have cross-regulatory influences that favour the predominance of one group over another. For example, IFN- γ (Th1) inhibits murine Th2 cell proliferation and Th2 cells inhibit cytokine synthesis of Th1 cells [231]. Th0 cells are thought to produce both type 1 and type 2 cytokines; it is unclear if they are an independent population or precursors of the Th1 and Th2 phenotypes. However, evidence suggests that Th1 and Th2 cells are derived from common precursors [232]. It has also been reported in murine *in vitro* studies that 'committed' cells can be influenced to change their cytokine profiles [233, 234].

The Th1/ Th2 paradigm has been demonstrated in diseases such as leishmaniasis and leprosy [235] where the pattern of cytokine expression is associated with different disease manifestations. An ineffective immune response in leprosy (lepromatous leprosy with florid skin lesions, high bacterial load and poorly formed granulomas) is typified by a Th2 type profile (IL-4) whereas a more resistant immune response (tuberculoid with limited skin lesions, paucibacillary disease and well formed granulomas) is characterised by a Th1 profile [235]. In general, Th1 responses promote protection against intracellular pathogens and parasites whilst Th2 responses promote protection against intestinal nematode parasites. However, non-infectious

diseases like asthma, Crohn's disease, organ transplantation and idiopathic pulmonary fibrosis are also associated with Th2 profiles. It is worth noting that, whilst the dominant cytokine in many of these diseases including asthma is IFN- γ , Th2 cytokines are significantly expressed. Consequently, the Th1/Th2 dichotomy does not simply imply a seesaw type down-regulation of one subset and up regulation of another. Indeed in TB we postulated that a small but significant IL-4 response, within the context of a dominant Th1 profile, sabotages protective host immunity [12, 236].

1.5. Putative mechanisms by which mycobacteria may subvert host immune responses

(i) Reactive oxygen and nitrogen intermediates

The dominant view has been that mycobacterial killing is mediated via reactive oxygen and nitrogen intermediates and oxidative effector molecules [237, 238]. Nevertheless, actual killing is difficult to demonstrate in human models and much of the data pertains to correlation with mycobacterial stasis. Furthermore, the exact mechanisms mediating these pathways and their importance in humans are unclear. However, mycobacteria possess a peroxidase/ phosphonitrate reductase system that may resist reactive host intermediates [239]. Mycobacteria are also able to alter their metabolism by switching to fatty acid oxidation thus reducing their oxygen requirements and the production of hemolysin may mediate mycobacterial escape into the cytoplasm [221, 240]. This mycobacterial compounds (glycolipids, LAM and sulphatids) may drive pathways that hinder the effective functioning of reactive intermediates. These mechanisms include inhibition of phagolysosome fusion and acidification [241], inhibition of antigen loading into CD1 proteins that requires a low pH environment [188]. How mycobacteria regulate and manipulate these pathways is unclear. Chan and co-workers suggest that a putative mechanism may involve mycobacterial antigens down regulating the transcriptional activation of IFN- γ inducible genes [242]. However, as discussed below, IFN- γ expression at the site of disease is prominent and mycobacterial antigens like ESAT-6 are potent inducers of IFN- γ . A detailed discussion about the putative role of IL-4 in relation to macrophage mycobactericidal functions is outlined in (vii) below.

(ii) Anti-mycobacterial peptides

CD8+ and NK T cells may directly kill mycobacteria; this is facilitated by perforin (forms a pore) and effected by granulysin (figure 1.3 [191]). Granulysin is a peptide present in the cytotoxic granules of NK cells and certain T cells and is directly cytotoxic (damages cell membranes) to intracellular bacteria including *M. tuberculosis* [191]. Another group of anti-bacterial peptides are the defensins; those found in neutrophil granules have been designated human neutrophil peptides (HNP). In *in-vitro* cultures defensins are lethal to *M.tuberculosis* [243], and in a murine model enhance mycobacterial killing and substantially reduce the required anti-mycobactericidal drug dose [244]. The protective role of other anti-mycobacterial peptides like the cathelicidins is under investigation. Nevertheless, the pathways that underpin the production of these peptides require further investigation. It is unknown whether host cytokines like IL-4 can modulate these pathways.

(iii) Defects in IFN- γ and Th1 pathways

It is clear that IFN- γ (produced by CD4, CD8 and NK cells) is important for mycobacterial destruction as mice and humans with genetic defects of these cytokines or their receptors are more susceptible to mycobacterial infections [245, 246]. Patients with MDR TB with failing treatment regimens have responded to aerosolised IFN- γ [104] and IFN- γ induction has been shown to be reduced in PBMCs from TB patients [29]. Consequently, sub-optimal IFN- γ responses and ensuing failure to activate macrophages has been postulated as a putative mechanism to explain mycobacterial persistence and disease progression. However genetic mutations of IFN- γ or its receptor in humans with pulmonary tuberculosis are rare in clinical practice [68] and most patients with active disease still have strongly positive DTH responses. Notably, *in vitro* models in humans have failed to show mycobacterial killing in monocytes following the addition of IFN- γ [101, 247]. In those with active disease rapid induction of a potent IFN- γ response to ESAT-6 is an almost universal consequence of infection [74]. Moreover, granulomas from TB patients have abundant IFN- γ and IL-12 [248]. Although low IFN- γ levels have been demonstrated in patients with active disease, these studies have invariably used peripheral blood cells [29, 78].

However, at the site of disease, the lungs, preliminary evidence indicates that levels of IFN- γ are high [7]. These data support the indispensable role of IFN- γ for protective immunity but also a hypothesis that it is not failure of protective Th1 responses but their deliberate induction to perpetuate immunopathology and concurrently their sabotage, which may explain the destabilisation of an effective host response.

(iv) TGF- β

IFN- γ mediated mycobactericidal activity of human macrophages is enhanced in the presence of TGF- β neutralising antibodies [249]. These data suggest that unbalanced TGF- β production is capable of suppressing successful host immune responses to tuberculosis by macrophage deactivation and T-cell inhibition. TGF- β may also be associated with tissue damage and fibrosis [131, 250]. But under what conditions does TGF- β facilitate immunopathology and /or mycobacterial proliferation? Existing data lend support to the hypothesis that Th2 cytokines, like IL-13, may drive TGF- β thereby sabotaging a balanced and coordinated host response (see section 1.7. for a more detailed discussion). Certain types of regulatory T cells also produce TGF- β (discussed below).

(v) Regulatory T cells and IL-10

It has been proposed that disease progression and susceptibility is facilitated by regulatory T cell (Treg) activity because peripheral blood T cells often respond less vigorously to antigens of *M. tuberculosis* than do cells from healthy contacts [251, 252]. Some of these are “anergic” TB patients who are skin-test negative and whose T cells secrete IL-10, but release little IL-2 or IFN- γ in response to *M. tuberculosis* [253-255]. Some T cells that secrete IFN- γ in the lungs of tuberculosis patients also secrete IL-10 [256], and might be the recently discovered Th1-like Treg that express both t-bet (transcription factor and Th1 marker) and Foxp3 rather than true Th1 cells [257, 258]. Other types of regulatory T cells that have been described include naturally occurring Treg [259, 260], Tr1 cells [253, 254], Th3 cells [251], regulatory NK T cells [261] and the list is growing. Naturally occurring Treg [260] and Tr1 (likely but not definitively proven) regulatory cells [253, 254] have been described in

association with tuberculosis. What these cells have in common is the ability to downregulate proliferative and pro-inflammatory functions of other cells; they generally produce IL-10.

IL-10, an anti-inflammatory cytokine, which inhibits human T cell proliferation, down regulates macrophage MHC class II expression, Th1 cytokine production [262], inhibits the microbicidal activity of macrophages [262] and down regulates CD1 expression on dendritic cells. Th3 type TGF- β secreting CD4⁺ lymphocytes, one of many Treg subtypes, may concurrently produce IL-10 and also small amounts of IL-4 [258]. However, the role of IL-4 in this context is unclear. In progressive disease, IL-4 causes increased, rather than diminished, immunopathology in BALB/c mice [14].

Collectively, these data indicate that regulatory T cells are present in patients with TB, but their importance remains unclear. It is possible that excessive early Treg function might result in a delayed development of protective Th1 activity, as in some malaria models [263]; however, the malaria parasite proliferates very fast, and a delay of a day or two can be critical, but this is less likely to be true for *M. tuberculosis*. Nevertheless, though a few TB patients are certainly anergic, most have tissue necrosis, a powerful IFN- γ response and recruitment of antigen-responsive T cells at the site of disease [7, 8, 264, 265]. Schwander and colleagues showed that there are high IFN- γ levels and strong proliferative responses in the human lung but the opposite occurs in the periphery [264]. This might suggest that in the chronic phase of progressive of tuberculosis, the extent of Treg activity is not the critical factor that determines outcome. Wherever the patients' immune systems lie on the Treg axis, they still have TB.

Already entertained are the possibilities of too much Treg in the very early phase of infection or too little Treg during progressive phase of disease because of the extensive immunopathology seen in TB. Another possibility is a wrong type of Treg response so that there is inappropriate facilitation of Th2 responses. Further studies are required to clarify which of these possibilities predominate and whether Treg responses represent cause or effect in TB. It also seems logical to determine, at the

levels of DCs, which antigens drive regulatory responses and T-helper differentiation in TB (discussed later).

(vi) Apoptosis

As already outlined, apoptosis may be beneficial for the host and facilitate mycobacterial killing (whereas necrosis is not). This may also explain why treatment with neutralising antibodies to TNF- α , which facilitates apoptosis, can cause reactivation of tuberculosis [153]; decreased expression of iNOS in the presence of anti-TNF might also be involved. It is therefore not unreasonable to suggest that defects in apoptosis pathways may enhance disease progression. Indeed, *M. tuberculosis* has mechanisms that tend to inhibit apoptosis. Mannose-capped lipoarabinomannan (Man-LAM) stimulates phosphorylation of Bad, a pro-apoptotic protein, and so inhibits apoptosis [266]. Another group has shown that Man-LAM inhibits apoptosis by a mechanism involving calcium-dependent signalling [267]. Recently cathepsins have been shown to modulate apoptosis in human pulmonary epithelial cells [268] though their role in TB has not been investigated.

Virulent *M. tuberculosis* seeks to evade apoptosis by causing infected human macrophages to release increased levels of soluble TNF receptors, leading to formation of inactive TNF- α -TNF receptor complexes [269, 270]. This strategy used by *M. tuberculosis* to avoid apoptosis is likely to be enhanced by IL-4, because IL-4 downregulates expression of TNF- α in human monocytes/macrophages [271, 272] and increases the release of soluble TNF receptors [271]. Whether IL-4 in TB causes a switch from apoptosis to necrosis, increases soluble TNF- α production and facilitates survival of *M. tuberculosis* requires further study.

Nevertheless, whether apoptosis is protective or deleterious remains contentious and there are conflicting data. However, there is evidence that apoptosis may facilitate deletion of protective T cell clones and IL-4 production is also linked to TNF-mediated lymphocyte apoptosis in Mycobacterium-reactive human T cells thereby contributing to immunopathology [26]. CD 30 expression and TNF mediated lymphocyte apoptosis were both down regulated by blocking IL-4 in this model. Collectively, these data suggest that IL-4 may modulate apoptosis in a cell-specific

manner. IL-4 may down-regulate beneficial macrophage apoptosis but enhance protective CD4+ T cell apoptosis.

(vii) Th2 cytokines (IL-4)

As already outlined, there is no dispute that Th1 cytokines are crucial for successful host defence against TB but neither are they sufficient to prevent disease progression. Discussed further in section 1.7 is the contention that *M. tuberculosis* induces a potent Th1 response to enhance immunopathology and hence propagation, and then deliberately undermines this response by deactivating protective macrophage functions. Evidence for the notion that IL-4 exacerbates immunopathology and augments the Koch phenomenon is also discussed. The putative mechanisms by which IL-4 subvert protective immunity is shown in table 1.3.

Table 1.3. Why is expression of IL-4 detrimental to immunity to TB?

- Inappropriate type of macrophage activation [273, 274] that may be less mycobactericidal
- Decreased expression of, and signalling through, Toll-like receptor 2 (TLR2).[275]
- Down regulated inducible nitric oxide synthase (iNOS) [276]; iNOS might be fundamental to the induction and maintenance of latency [277].
- IL-4 favours necrosis rather than apoptosis, which is mycobactericidal by down regulating the expression of TNF- α in human monocytes/macrophages (discussed under apoptosis in section 1.5 above)
- In mixed Th1 + Th2 lesions, IL-4 facilitates TNF- α toxicity (discussed in detail under immunopathology in section 1.7.4.)

1.6. IL-4 and IL-4delta2 (IL-4δ2)

1.6.1. Biological properties of IL-4

Compared to the effects of Th1 cytokines, the prototype Th2 cytokine, IL-4, antagonises IFN- γ production, inactivates macrophages by down-regulation of CD14 and Fc γ receptors [278, 279], down regulates IL-2 receptor expression in T lymphocytes [280] and inhibits transcription of the IL-2 gene [281]. Notably, IL-4 causes an alternative form of macrophage activation [274]. Consequently, cytokines like IL-4 have the ability to circumvent protective host immune responses. IL-4 also enhances B cell MHC Class II expression, growth and differentiation; production of non-complement-fixing IgG isotypes (IgG1 in mice and IgG4 in humans); induces expression of CD23 (low affinity IgE receptor) and together with IL-13 facilitates IgE class-switching [282]. IL-4 acts synergistically with other Th2 cytokines like IL-13 that share some of the properties of IL-4, IL-5 which enhances eosinophil production in the bone marrow [282].

1.6.2. Th2 differentiation and source of IL-4

Until recently, it has been widely accepted, based on several studies that IL-4, itself, stimulates the development into a type 2 phenotype by binding to IL-4R α . This notion implies that differentiation of naive CD4 T cells to Th2 cells requires a source for the IL-4 that would prime the T cell response. There are several possible sources including mast cells, basophils, eosinophils, and NK T cells, though none in particular have been found to be necessary for the development of an in vivo IL-4 response [283]. However, it is now becoming clearer that an IL-4 independent mechanism of Th2 differentiation exists. As already discussed (section 1.4; DCs), microbes by ligation of pattern recognition molecules on APC or by provoking tissues to release mediators, can modulate Th1/ Th2 differentiation. Thus, during antigen presentation to T cells, in addition to association of the TCR and MHC II proteins on APC (signal 1) and ligation of co-stimulatory molecules (signal 2), differentiation of T cells is also initiated (signal 3). Indeed, protein extract derived from the helminth *Schistosoma*

mansoni induced the development of Th2 cell-promoting effector DCs (DC2) that promote the development of Th2 cells via the enhanced expression of OX40 ligand, whilst toxin *Vibrio cholerae* induced development of Th1 inducing effector DCs (DC1) via an OX40 ligand-independent, still unknown mechanism [284]. Notably, immature DCs derived from BCG-infected precursors acquire a mature phenotype, produce IL-10 and IL-4, and direct a Th2-like immune response [174].

Collectively, these data indicate that microbial compounds can govern the molecular basis of Th1 [285] Th2 [174, 284] polarization via DCs. It remains unclear whether different DC lineages drive the final commitment of T cells or whether the same subset of DCs can promote either a Th1 or a Th2 response [286-289]. Factors that may determine which Th response is generated include the nature and load of micro organism, type of pattern recognition receptors engaged, factors released by tissue and inflammatory cells (damage signals), physical interactions with other cell types, death of infected cells and duration of the TCR engagement [286].

In addition to those previously mentioned other cell types that produce IL-4 include CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, B cells, eosinophils, basophils, DCs and sometimes macrophages (when exposed to virulent Beijing strains) [290]. Finally it is worth mentioning that attention has been focused recently on the neglected observation that IL-4 can be involved in driving Th1 responses [291] and CTLs [292]. This can be IL-4 administered externally or IL-4 from CD11b⁺CD11c^{lo} APCs, which are present at an early stage of Th1 response induction [293]. IL-4 present at later stages downregulates Th1 responses [291], and this may be happening in TB, where at least some of the Th2 cytokines are coming from T cells [19, 29]. However, none of the literature on this topic is interpretable, because the workers involved did not distinguish between IL-4 and IL-4 δ 2. It is possible that the “IL-4” involved in a very early stage of Th1 differentiation is in fact IL-4 δ 2.

1.6.3. The IL-4 receptor and associated signalling pathways

The IL-4 receptor component, IL-R α (CD 124) pairs with other components to form receptor heterodimeric complexes. IL-4R α can combine with the γ c chain to form the

receptor for IL-4 (predominates on haemopoietic cells; designated type I IL-4 receptor). Association of IL-4R α with IL-13R α 1 constitutes a common receptor for both IL-4 and IL-13 (found on non haemopoietic and haemopoietic cells; designated type II IL-4 receptor), and IL-13R α 2 alone can bind IL-13 and forms a 'decoy' receptor [294]. Two known downstream signalling pathways, the Stat-6 pathway and those mediated by binding to the insulin–interleukin-4 receptor (I4R) motif, alone or in combination, modulate the varied effector functions of the IL-4R α (gene expression, proliferation and differentiation). The most well-known domain adaptors that bind to I4R, though other have also been described [294], are members of the insulin receptor substrate family (IRS family; IRS 2 being the most prominent) and Shc, which mediate growth promoting and regulatory signals through IL-4R α . Downstream signalling of these pathways (IRS and Shc) is mediated by the phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades, respectively.

Binding of IL-4 to the receptor initiates a cascade of kinase-mediated phosphorylation reactions that facilitates the binding of the transcription factor Stat6 to the cytoplasmic domain of IL-4R α . Stat6 undergoes dimerization and translocation to the nucleus, where it activates the transcription of IL-4 and IL-13-responsive genes [294]. Stat-6 also induces GATA-3 and c-Maf, which have similar effects on IL-4 and IL-13 transcription, as opposed to STAT-4 and t-Bet, which are Th1 inducing transcription factors [295]. There are also pathways (Shp-1), which negatively regulates Stat 6-mediated receptor signalling [294]. Notably, studies using mice that were deficient for IRS-2, Stat6 or both revealed that both Stat6 and IRS-2 contribute to naive CD4 T-cell proliferation in response to IL-4 [296].

1.6.4. Regulation of IL-4

1.6.4.1. Transcriptional regulation and mRNA stability

As already outlined, ligation to type I and type II receptors, mediates the activation of complex Stat6 dependent and independent signalling pathways, which culminate in transcription of IL-4 responsive genes. Presumably, the flexibility in the signalling

cascade facilitates the modulation of IL-4 effects in different tissues, though the regulation of these pathways is incompletely understood. Steady state mRNA levels are dependent on transcription rates and mRNA stability (rates of degradation). Stability of mRNA is influenced by many of its components, including the 5' cap, the 3' poly-(A) tail, sequences within the 3' untranslated region (UTR), sequences within the 5' UTR and sometimes motifs within the protein-coding sequence itself.

However, more recently interest has focussed on the MAPK pathway and its effect on cytokine regulation. So far 3 distinct MAPK pathways have been described in mammalian cells (extracellular signal related kinase, ERK 1 & 2; c-Jun-terminal kinase 1 and p38 α MAPK); the p38 MAP kinases play an important role in cellular responses to external stress [297-299]. mRNAs for TNF- α and IL-1 contain AU binding proteins in the 3' UTR, which block translation and facilitate rapid turnover of transcripts [298-300]. Activation of p38 α MAPKs results in phosphorylation and release of the AU rich proteins allowing translation and secretion of cytokines like TNF and IL-1 to occur. Consequently, inhibitors of p38 MAPK (pyridinyl imidazole class of compounds) prevent translation, cause rapid turnover of transcripts and decrease the half life, and therefore have anti-inflammatory effects. It is worth noting that AU binding proteins are one of many factors that regulate mRNA stability and p38 independent pathways of AU protein phosphorylation have been described [301-303]. There are no data on whether the half-life of IL-4 is prolonged in TB and whether the transcriptional regulation is MAPK dependent.

1.6.4.2. Soluble IL-4 receptors (sIL-4R)

The extracellular domain of the human IL-4R, soluble IL-4R (sIL-4R), which is formed after proteolysis of the membrane-bound receptor [304] neutralises IL-4 *in vitro* [305] and *in vivo* [306]. Soluble cytokine receptors can have inhibitory effects because they block access to the receptor on the cell membrane. Alternatively, they can enhance cytokine effects by acting as a carrier, prolonging half-life, and releasing cytokine to the membrane receptors in a delayed fashion. This had been described for soluble IL-6 receptors [307] but is also true of soluble IL-4 receptors. Indeed, no IL-4 bioactivity could be determined in supernatants of T cells activated in the presence of

IL-4 for 6 days. In contrast, the same cultures carried out in the presence of sIL-4R showed marked IL-4 bioactivity [308]; implying that IL-4/sIL-4R complexes liberated free IL-4 even during prolonged culturing.

Contrary to expectation, sIL-4R alpha levels are increased in BAL from stable asthmatic children compared with nonatopic controls [309] and also in subjects with allergic rhinitis [310]. These data suggest that the serum half-life of sIL-4R is higher than that of IL-4 and it might therefore be a simple and more stable surrogate marker of IL-4 activity. In summary, sIL-4R is both an antagonist and also a protective molecule that can enhance agonistic functions of IL-4 [308]; low concentrations of sIL-4R enhance IL-4-driven effects and high concentrations neutralized IL-4. There are no studies examining the levels of either membrane-bound or soluble IL-4R α in tuberculosis.

1.6.4.3. Possible role of natural antibodies to IL-4

The same principal may also be true for antibodies to IL-4. Some antibodies protect the IL-4, and act as carriers [311]. The same idea has been applied to prolong the life of recombinant IL-4 in the mouse [312]. It is well known that even normal individuals can have antibodies to cytokines [313]; these can be of high affinity. Interestingly, natural antibodies to human IL-4 have not been investigated [313]. It is intriguing to speculate that *M. tuberculosis* might act as an adjuvant and drive production of natural antibodies to IL-4, which then prolong the half life and activity of IL-4. Whether developing country subjects with high serum IL-4 levels, detectable by ELISA, also have high circulating antibody to IL-4 requires investigation.

1.6.5. IL-4 δ 2 (IL-4 δ 2)

In addition to the regulatory mechanisms already described (transcriptional, sIL-4R and possibly natural antibodies to IL-4) IL-4 δ 2 may function as a supplementary regulator of IL-4. IL-4 δ 2 is a naturally occurring and only proven splice variant of IL-4, which lacks exon 2 (48 base pairs) [33, 314] resulting in part deletion of the mature protein (48 bp coding for amino acid residues 22 to 37 with consequential omission of

a double stranded anti-parallel β -sheet normally found in IL-4) [33]. IL-4 δ 2 mRNA found in primates, rabbits, woodchucks, cows and mice [33, 315, 316]. A murine IL-4 mutant protein with C118 deletion abrogates STAT6 phosphorylation and IL-4-induced IgE production *in vitro*; furthermore, modifications in the C-terminal region of IL-4 produce mutants that bind to the IL-4R α with high affinity but do not induce cellular responses [317]. Furthermore, alternative splicing of mRNA encoded by a single gene is a well described regulatory mechanism that controls gene expression and generates structural and functional diversity of proteins [318, 319], which may be differentially expressed in certain tissues, or during different stages of development, or cell activation [320-322]. Indeed, recombinant IL-4 δ 2, cloned and expressed in yeast, functions as a natural competitive antagonist of human IL-4 (analogous to IL-1 and the IL-1ra), binds to the IL-4R α chain only, and may be the dominant form of IL-4 mRNA in human alveolar lavage cells where it is associated with scleroderma-induced interstitial fibrosis [44, 314, 323]. Recombinant human IL-4 δ 2 appears to inhibit IL-4-induced human T cell proliferation, which is greatest at lower concentrations of IL-4 [314, 324]. Moreover, in B-cells IL-4 δ 2 blocks IL-4-induced IgE synthesis and CD23 expression. By contrast IL-4 δ 2 is possibly an agonist on human fibroblasts [44]. IL-4 δ 2 is also expressed in PBMC samples from normal human donors, thymic samples and endobronchial biopsies [26, 325, 326]. The relative proportion of IL-4 δ 2 to IL-4 mRNA varies between different cell sources in the same donors and changes with stimulation [325, 327].

Final proof that the IL-4 δ 2 protein is produced *in vivo* has not yet been obtained and the clinical significance of IL-4 δ 2 remains unknown. Meaningful evaluation of existing work on IL-4 in man is challenging, as the antibodies used in flow cytometry or ELISA assays may bind either or both cytokines. The agonist IL-4 and the antagonist IL- δ 2 are easily distinguished by RT-PCR [328], but most published work uses primers that will amplify both cytokines. IL-4 δ 2 has been studied in human scleroderma [44], gastritis [329], transplantation [330] and bronchial asthma where IL-4 δ 2 rises very little but IL-4 mRNA itself rises 1000x more [331]. IL-4 δ 2 mRNA expression in TB is discussed in section 1.8. below. Collectively, the observations in bronchial asthma and allergic rhinitis demonstrate the therapeutic potential of IL-4 mutant protein receptor antagonists, anti-IL-4 antibodies and sIL-4R.

1.6.6. Soluble CD30 (sCD30) and surrogate markers of Th2 differentiation

In addition to IL-4, IL-5 and IL-13 surrogate markers of a Th2 phenotype include expression of the chemokine receptors CCR4, CCR8 and CCR3 on type-2 cells [332], MDC/CCL22 (macrophage-derived chemokine), human IgE and IgG4, the IgE receptor (CD23) expressed on B cells, and soluble CD30 (sCD30) and membrane bound CD30. However, some of these markers are transiently expressed and none are exclusively expressed on Th2 cells alone.

CD30 is a widely distributed (CD4, CD8, $\gamma\delta$ T cells, B cells, NK cells, CD4RO+ memory cells) membrane glycoprotein belonging to the TNFR superfamily; the extracellular domain is a binding substrate for CD30 ligand. The cytoplasmic domain binds TRAF (TNF receptor-associated factor) which mediates signal transduction [333]. Downstream kinase mediated phosphorylation reactions activate NF κ B, though other pathways have also been described [333]. CD30 expression is low in healthy donors; only about 0 to 2% of PBMCs express CD30 [333]. sCD30 is derived from proteolytic cleavage from the membrane bound form. The functional relationship between sCD30 and membrane CD30 is uncertain.

Several previous studies, demonstrating increased CD30 or sCD30 in Th2 conditions, suggested that this was a Th2-specific marker (reviewed in [333]). However, the emergence of further data suggested that CD30 is present on activated T cells of Th0, Th1 and Th2 profiles. Based on flow cytometric studies it was shown that Th2 skewed-cells show higher receptor density and these are present on the cell surface longer than on Th1 cells [334]. Ligation of the CD30 receptor induces increased co-expression of the IL-4 receptor [335], whilst blockade of CD30–CD30 ligand interaction inhibits development of Th2 cells, lowers the production of IL-4 and IL-5, and shifts production toward the Th1 profile [336]. Functionally therefore high sCD30 and CD30 receptor expression is preferentially active during Th2-dependent reactions. Nevertheless, the pleiotropic effects of CD30 are also cell sub-type dependent; for example CD30 receptor ligation induces IFN- γ in CD45RO+ memory T cells and increased IL-8 in $\gamma\delta$ T cells [333].

In addition to facilitating cell differentiation CD30 mediated signalling is important in several different activities of T cells including anti CD3 induced proliferative responses [337, 338], apoptosis [338] and induction of HIV gene expression in infected T cells [339]. *M. tuberculosis* sonicate up regulates CD30 expression and induces IL-4 dependent TNF mediated apoptosis in peripheral blood lymphocytes from healthy donors [26]. sCD30 levels are elevated in the serum of adult [340] and paediatric [341] subjects with active TB compared to community controls and the level decreases with treatment [340]. Differential expression of sCD30 in alveolar lavage fluid and peripheral blood compartments has not previously been studied in tuberculosis and is discussed further in relation to data presented in chapter 5.

1.6.7. Measurement of IL-4 and IL-4δ2

Difficulties in measuring IL-4, which may account for the failure of earlier studies to find IL-4, are discussed in detail in chapter 2 (section 2.1.1.) Briefly, there are a number of technical reasons why IL-4 detection is difficult (short half life, activity at low concentrations, rapid internalisation, failure to distinguish between free and bound IL-4). Notably, the interpretation of existing data is undermined by the failure to take into account the natural antagonist of IL-4, IL-4δ2. Furthermore, the use of pre-stimulation protocols to detect IL-4 is Th1 biased and unphysiological. To overcome these problems investigators have turned to PCR. However, conventional PCR has its own set of technical hitches. These problems and the development and validation of an assay to overcome them are discussed in Chapter 2.

1.7. The Th2 response and immunity to tuberculosis

Encouragingly, a few TB vaccine candidates are now entering clinical trials. However, some workers are sceptical because these vaccines have been designed with little reference to the immunopathogenesis of TB, and without understanding why the existing vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is failing in developing countries. This is a high-risk strategy because the new vaccines might fail for the same reasons. It will be tragic if we have been designing vaccines based on

studies of patients in Europe and the USA, and consequently the vaccines fail to elicit protection under the different immunological conditions of developing countries.

The following section first outlines the nature of the protective Th1 response. Thereafter the available data pertaining to IL-4 expression in TB are reviewed. The hypothesis that the production of IL-4, superimposed on a Th1-cell response, is able to convert it from a protective response to an immunopathological one that is essential for the pathogenesis and spread of TB, is also discussed. Furthermore, the hypothesis that progression from latency to disease will correlate with a decrease in IL-4δ2 (antagonist) relative to IL-4 (agonist), is reviewed. It is suggested that vaccines that only induce Th1 might work in strict pathogen-free laboratory conditions, but vaccines for humans (post-exposure) will need to focus on immunoregulation and downregulation of pre-existing IL-4 responses. Finally, based on available evidence, it is argued that successful vaccines for countries close to the equator might need to eliminate the pre-existing IL-4 component. These ideas and the hypothesis have been reviewed in detail [12, 236, 342].

1.7.1. Immunity; is the size of the Th1 response the critical factor?

Th1 activity and TNF-α are essential for immunity to *M. tuberculosis*. Genetic defects in receptors for IFN-γ or IL-12 lead to increased susceptibility [343, 344] and neutralising TNF-α with therapeutic monoclonal antibodies can lead to reactivation of latent disease [153]. Therefore, at first glance, it seems logical to seek vaccines that maximise the Th1 response. However, it is logical only if TB occurs because of a failure to generate a Th1 response or the Th1 response does not develop fast enough. That there are prominent Th1 responses in TB, and further enhancing this response is unlikely to be helpful, is discussed in relation to the data presented in chapter 5.

1.7.2. IL-4 and IL-4 δ 2 in tuberculosis infection and disease

1.7.2.1. Active tuberculosis: Whole blood and peripheral mononuclear cells

In the last decade, studies about the expression of IL-4 in TB have been conflicting and the issue has remained controversial. Furthermore, the findings of poor proliferative responses and low IFN- γ levels in whole blood dominated reports and the conflicting IL-4 data were ignored. Surcel *et al* found increased production of IL-4 by ELISPOT, in response to stimulation of PBMCs with mycobacterial antigens in UK donors with TB, but reduced proliferation and no change in IFN- γ producing cells when compared to control subjects [18]. Similarly, Schaaf and colleagues detected IL-4 mRNA by RT-PCR in most N. American TB patients but none in controls [345]. Remarkably, a number of reports emerged from developing countries (Argentina, Venezuela and China [a large study of over one hundred patients]) indicating that IL-4, but not IFN- γ , was elevated to the extent that it was detectable by ELISA in the serum of patients with TB compared to controls [20, 22, 346]. Such high levels of IL-4 are not detected even in asthma patients in the UK. Other studies also from developing countries (Mexico, The Gambia and India) using flow cytometry after intracellular cytokine staining (ICCS), showed that PBMCs stimulated by TB antigen produced significantly greater levels of IL-4 compared to control subjects [21, 24, 29, 347].

More recent data suggest that in addition to Th1 cytokines, there is indeed an IL-4 response in human TB both in Europe [17, 19] and in Africa [340, 348]. van Crevel *et al* showed that IL-4 was elevated (with little difference in IFN- γ levels) in PMA/calcium-ionophore stimulated CD4 and CD8 cells from Indonesian patients with tuberculosis compared to healthy controls [19]. Notably, the primers and immunoassays used in all the studies described so far did not distinguish between IL-4 and IL-4 δ 2. Seah *et al*, using a semi-quantitative nested RT-PCR assay, showed that IL-4 and IL-4 δ 2 production is increased in British subjects with pulmonary TB, and correlates with immunopathology [17, 349]. Furthermore, Sousa and colleagues found elevated levels of Th2-driven IgG4 antibodies, in high titres, in the Yanomami Indians of the Brazilian Amazon [350]. TB patients also have several IL-4-dependent

phenomena, including IgE antibody to *M. tuberculosis* [351], increased expression of DC-specific intercellular adhesion molecule (ICAM)-grabbing non-integrin (DC-SIGN) [161, 163], and antibody to cardiolipin [352]. Collectively, the investigators of these studies concluded that patients with tuberculosis had a Th2 type response in peripheral blood

By contrast, other studies failed to find significantly raised IL-4 levels in subjects with tuberculosis compared to healthy volunteers [25, 27, 30, 32, 264, 353-355]. Investigators from the USA using RT-PCR [27, 28] and immunohistochemistry [354] failed to show increased expression of IL-4 mRNA in donors with active TB. Verbon and co-workers from the Netherlands failed to demonstrate detectable IL-4 by ELISA in 84 subjects with active TB [32]. Other investigators from developing countries (Mexico, China) using RT-PCR [30, 355] and ELISA [264] also failed to find elevated IL-4 expression in subjects with tuberculosis.

The conflicting data may be attributable to a series of technical problems related to the measurement of IL-4 (outlined in section 1.6.7 but discussed in detail in chapter 2). Studies which detected elevated IL-4 mRNA levels compared to controls [17, 186] and those that failed to do so [27, 28, 30, 355] used conventional semi-quantitative RT-PCR protocols. The unreliable quantification and other drawbacks of this technique are discussed in detail in chapter 2.

Taken together two conclusions can be drawn from the studies that have been reviewed. Firstly, there is reasonable evidence to suggest that IL-4 levels are up regulated in TB, though there are conflicting data. This suggests that a carefully validated approach, that takes into account various technical and other factors, needs to be developed for the reliable detection of IL-4 (discussed in detail in chapter 2). Secondly, IL-4 levels in TB patients close to the equator are generally raised, to the extent that it is detectable by ELISA (the validity of these findings and possible adjuvant effect of helminth infection is discussed in detail in section 1.7.5.2 below). Nevertheless, whether IL-4 represents cause or an immunological bystander effect requires further clarification. The studies reviewed here are discussed further in relation to the data presented in chapter 5.

1.7.2.2. Active tuberculosis: Broncho-alveolar lavage

Despite most antigen-specific cells being located at the site of disease, usually the lung, most studies have acquired samples from peripheral blood due to ease of sample acquisition. Consequently, there have been few studies that have examined cytokine profiles in lung lavage [24, 264, 265, 356-359]. Remarkably, in relation to a disease that kills ~3 million humans annually there are only 2 published studies comparing compartment-specific (blood vs lung) cytokine profiles in active TB [24, 264]. Older studies (prior to the year 2000) using either in situ hybridisation, cyto-immunochemistry or immuno-assays failed to detect IL-4 in BAL fluid [357-359] or in TB antigen stimulated alveolar lavage cells [264]. By contrast, other studies [24, 265, 356] show raised IL-4 levels in donors with pulmonary TB. Mazzarella and colleagues, using ICCS in PMA stimulated alveolar lavage cells from Italian patients found that the percentage of IL-4-secreting cells averaged 14 % in samples from cavitary disease, but only 2–3% in samples for noncavitary disease [356]. Sharma and co-workers, using similar methods, found IL-4 to be upregulated in alveolar lavage cells in Indian patients with tuberculosis [24].

Collectively the data regarding IL-4 expression in BAL fluid are conflicting. The reasons for this and the study findings presented here are discussed in detail in relation to the findings presented in chapter 5.

1.7.2.3. Contacts of patients with TB

Is expression of IL-4, or of the potential antagonist IL-4 δ 2, increased in latent TB, and if so, how does this differ from what happens in those in whom the disease progresses? Recent studies have focused on healthy individuals in The Gambia and Ethiopia, whose peripheral blood mononuclear cells produce IFN- γ responses to the TB-specific antigen, ESAT-6. These individuals have latent TB and it has emerged that they have significantly raised levels of mRNA encoding IL-4 δ 2 in their unstimulated PBMCs [360, 361]. In The Gambia, expression of IL-4 is also increased [361]. Furthermore, a study of healthcare workers in Portugal has revealed that those individuals whose peripheral blood mononuclear cells synthesised IL-4 in response to *M. tuberculosis in vitro* went on to develop overt TB within 2–4 years [15]. The

methods used, however, will not have distinguished between IL-4 and IL-4 δ 2. It remains unclear whether a high IL-4, rather than IL-4 δ 2, predisposes to disease; the findings of these studies are further discussed in relation to the data presented in chapter 5 and chapter 7.

1.7.3. Bovine TB and mouse models

Infection of cattle with *Mycobacterium bovis* generates both IFN- γ and IL-4 responsiveness to PPD and to ESAT-6 [362]. Similarly, the extent of lung pathology correlates with the production of IL-4 by peripheral blood mononuclear cells *in vitro* in response to PPD [363]. Interestingly, when cattle were immunised with a DNA vaccine, and then 3 weeks and 6 weeks later, vaccinated again with the protein encoded by the DNA vaccine emulsified in oil, they were more susceptible than non-immunised controls. This increased susceptibility correlated with an increased IL-4 response to subsequent challenge [363].

The relevant mouse models are those in which, as in humans, IL-4 production is observed during the progressive phase of the disease [364, 365]. These models of immunopathology are discussed further in the next section. Nevertheless, there are also knockout models that show no effect of IL-4 or STAT-6 on disease progression and immunopathology [366, 367]. These discordant findings are discussed further in relation to data presented in chapter 5.

1.7.4. Th2 cytokines and immunopathology

Various cell types, cytokines and other factors (toxic effect of mycobacteria, Koch phenomenon, Schwartzman reaction, MMP dysregulation etc) have been implicated in the genesis of TB- related immunopathology (these have been discussed in section 1.4.2 and recently reviewed in detail [31]). What is discussed here is the relationship between immunopathology and Th2 cytokines.

1.7.4.1. Necrosis and cavitation

The data demonstrating the presence of Th2 cytokines in human tuberculosis have already been reviewed (section 1.7.2). In TB-infected mice, susceptibility to toxic effects TNF- α injected into the footpads coincided temporally with the emergence of

Th2 cytokines in the lungs [368]. IL-4 may also regulate TNF- α mediated enteropathy in *Trichinella spiralis* infection [369] and necrosis in a schistosomiasis model coincides precisely with the superimposition of Th2 response on an existing Th1 pattern [370]. Murine (BALB/c) models of TB showed high IFN- γ levels early in infection, with increased levels of IL-4 during the chronic phase of infection, which was characterised by progressive fibrosis and necrosis [364, 371]. The Th2 response may exacerbate tissue damage by enhancing the pathological effect of TNF- α [368]. More recent evidence, from IL-4 gene knockout in BALB/c mice, showed an absence of TNF- α -mediated toxicity following TNF- α challenge in the absence of IL-4 [14].

Collectively these murine data suggest that under the influence of superimposed Th2 cytokines TNF- α is toxic in dominantly Th1-mediated lesions. It is not unreasonable to suggest that a similar mechanism operates in humans. This observation can explain the paradoxical role of TNF- α in TB. Indeed, TNF- α is crucial for protection against TB in animals [372] and in humans [153]. Nevertheless, in progressive human disease, as in progressive TB in the BALB/c mouse [14], it is toxic, and drugs that shorten the half-life of the mRNA encoding TNF- α lead to weight gain and symptomatic relief [373]. Because IL-4 levels are raised in progressive human disease, it is likely that, as in mice, IL-4 is involved in the toxicity of TNF- α . Examples of interactions between IL-4 and TNF- α in other diseases, and possible mechanisms for proinflammatory interactions between these cytokines, are described in table 1.4

In humans with TB studies of lavage fluid show the presence of IL-4 producing Th2 lymphocyte subsets in those with cavitory tuberculosis [356] and the presence of Th1 cytokine profile in non-cavitory disease [357]. Similarly expression of IL-4 in peripheral blood lymphocytes correlates with cavitory disease [19].

Table 1.4. Synergism between interleukin-4 (IL-4) and TNF- α , and exacerbation of immunopathology in tuberculosis.

Putative mechanisms of TNF- α mediated toxicity in IL-4 containing lesions.

- Upregulation of inflammatory cell recruitment by induction of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells [374].
- Induction of cortisol-resistance in lymphocytes [375].
- TNF- α mediated apoptosis is observed in TB antigen driven CD30+ T cells in an IL-4 dependent manner [26].

Examples of TNF- α mediated immunopathology in mixed Th1/ Th2 lesions

- Th1/Th2 granulomata of Schistosomiasis are sensitive to systemic cytokine release triggered by lipopolysaccharide (LPS) [376, 377]
- Gut immunopathology due to *Trichinella spiralis* infection is facilitated by TNF- α in a mixed Th1/ Th2 response (no immunopathology observed in IL-4 knockout mice [369]).

In other murine knockout models of immunopathology induced by mycobacteria, γ/δ T cells [201] α/β positive T cells, IL-12, IFN- γ and TNF- α [378, 379] also seem to be essential for necrosis of granulomata. It has been suggested that caseous necrosis is a protective mechanism to reduce the logarithmic growth of the organisms [105, 210]. However, it is apoptosis rather than necrosis that destroys *M. tuberculosis* (discussed below), so it is intriguing to speculate that *M. tuberculosis* has evolved components that exploit the host's tissue-damaging protective responses by driving a Th2 response and necrosis (summarised in figure 1.5). It is interesting that particularly virulent Beijing strains of *M. tuberculosis* cause human monocytes to express IL-4 and IL-13 [290].

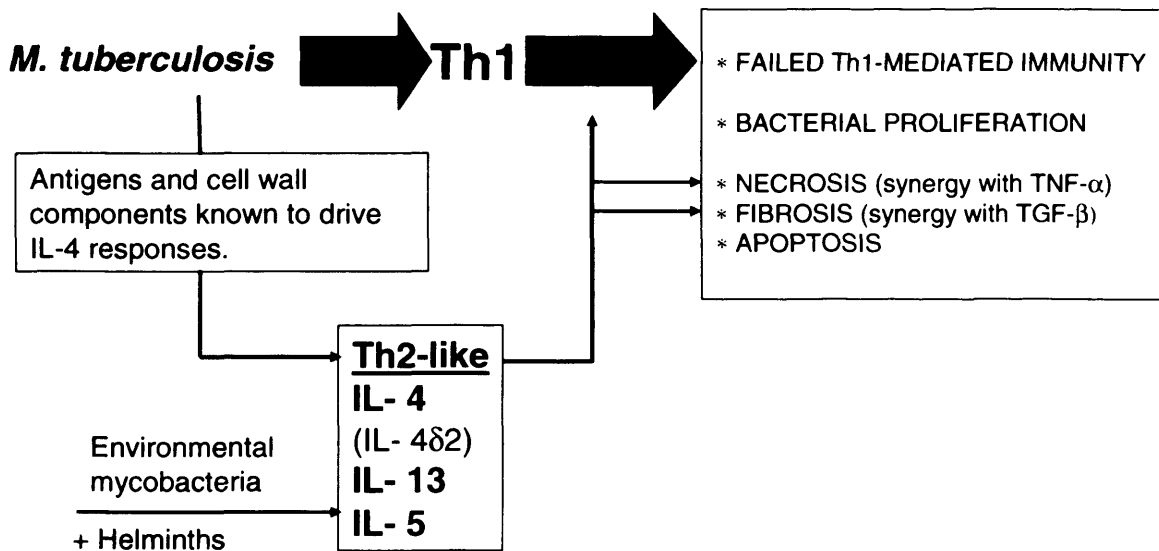


Figure 1.5. The roles of the “subversive” Th2-like component of the immune response in progressive human tuberculosis. This Th2-like IL-4 response, modulated by IL-4δ2, may be primed by environmental mycobacteria and helminths, and is most striking in developing countries. Following exposure to *M. tuberculosis*, certain cell wall components and protein antigens are then able to drive and enhance this IL-4 response, which may contribute to deactivation of macrophages, and to necrosis and fibrosis.

1.7.4.2. Fibrosis in tuberculosis

TNF-α , TGF-β and the Pathogenesis of fibrosis

Extensive deposition of ECM occurs in the guinea pig model of tuberculosis [380]. Available data suggest that TGF-β together with TNF-α plays a key role in the formation of the fibrous wall that encapsulates the tuberculous granuloma [130-132]. The importance of TGF-β with respect to pulmonary fibrosis has been established in human [250, 381] and animal models [382, 383]. However, it seems that TGF-β, while necessary, is not sufficient for fibrosis. The importance of other molecules like TNF-α in lung fibrosis is illustrated by models of bleomycin and hypersensitivity

pneumonitis where anti-TNF- α antibodies ameliorate lung fibrosis [384, 385]. The potential role of other cell types in lung fibrosis has recently been reviewed [31].

Within the context of TB and lung fibrosis, two paradoxes immediately arise. The first is the presence of increased ECM at the periphery of the granuloma but dissolution in the central part of the granuloma. Possible explanations include a cytokine gradient within the granuloma (low IFN- γ levels but high TNF- α in the central part of the granuloma) [386] and selective binding of cytokines to glycosaminoglycans in different parts of the granuloma [387]. The second paradox is the presence of fibrosis where there are high IFN- γ levels, which down regulate TGF- β and production of collagen by fibroblasts [388, 389]. The answer may lie in the significant Th2 response that develops, parallel to and within the framework of a Th1 response.

Role of Th2 cytokines in fibrosis

IL-4 and IL-13 are profibrotic and enhance collagen production by fibroblasts [389]. Mice when infected with saprophytic mycobacteria only develop peribronchial and interstitial fibrosis when primed for IL-4 [364]. As already outlined, even a single epitope (16 amino acids) inducing a Th2 response can drive fibrosis in a murine model of tuberculosis [16]. In all human diseases characterised by marked pulmonary fibrosis there is expression of type 2 cytokines [systemic sclerosis, idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis, chronic lung allograft rejection (reviewed in [390]). The same is true in the bleomycin murine model and in schistosomiasis, where fibrosis and tissue remodelling do not occur if induction of type 2 cytokines by the ova is blocked by pre-immunisation with ova + IL-12 [391]. These observations have led to the “type 2 cytokine hypothesis of fibrosis” [390]. This paradigm implies an initial Th1 type response to antigenic challenge, followed by a Th2 response that seeks to ‘wall off’ or isolate a persistent antigen from the host [377, 392, 393]. *M. tuberculosis* may be exploiting this host response to remain immunologically ‘sealed off’ in fibro-caseous foci.

The Th2 phenotype causes fibroblast activation and collagen deposition in human

[394, 395] and animal models [383, 396]. Collagen synthesis in granulomata is stimulated by Th2 cytokines (IL-4, IL-13 and IL-4 δ 2) and reciprocally inhibited by Th1 cytokines like IFN- γ and IL-12 [391, 397]. Moreover granulomatous inflammation and fibrosis is significantly reduced in Stat6 $^{-/-}$ mice [398]; IL-4 and IL-13 are the major activators of Stat6. Fibrosis is particularly pronounced in mice over-expressing IL-13 [390]. This cytokine, like IL-4 and IL-4 δ 2 [44], activates fibroblasts and promotes collagen formation. However IL-13 also drives expression and, activation of TGF- β [390]. Significantly the increase in expression of type 2 cytokines in human TB extends to IL-13 which like IL-4 and IL-4 δ 2, is increased 80-100 fold [17, 349].

Collectively, the data presented in the previous 2 sections demonstrate a strong association between Th2 cytokines and immunopathology. This is significant because fibrosis, necrosis and lung remodelling is a major cause of lung morbidity worldwide, it enhances disease progression and is the principle mechanism by which disease spread occurs.

1.7.5. Th2 cytokines and implications for vaccine design

1.7.5.1. Antigens that drive IL-4 and implications for vaccine design

Table 1.5 lists the vaccines currently entering clinical trials. Notably, vaccines that down regulate IL-4 (though they may drive Th1) are active therapeutically in murine TB whereas those that exclusively augment Th1 responses may actually worsen the disease [149]. Details of these vaccines, the putative mechanisms by which they may enhance protective responses, including the possible induction of IL-4 δ 2, is discussed further in relation to data presented in chapter 7.

Table 1.5. Types of new tuberculosis vaccine that are entering clinical trials (81)

1) Boost responses to antigen 85 A or B (Ag85)

- Recombinant BCG over-expressing Ag85.
- Boost response to Ag85, more than BCG does normally.
- Modified vaccinia virus Ankara expressing Ag85 [399, 400]. This might be given to subjects who have already received BCG in infancy to boost both CD4+ and cytotoxic CD8+ responses to Ag85.

2) Boost responses to *M. tuberculosis* proteins recognised by healthy PPD+ donors.

- Recombinant fusion-protein. Boosts CD4+ and CD8+ T-cell responses to Mtb72F, a 72-kDa polyprotein consisting of Mtb32 and Mtb39
- Fusion protein of ESAT-6 and Ag85B

3) Boost BCG-induced CD8+ T-cell responses.

- Recombinant BCG expressing listeriolysin [401]. The listeriolysin lyses the phagosome membrane. Then BCG can enter the cytosol for processing by the MHC class I pathway and presentation to CD8+ T cells.

4) Boost responses to the "common" mycobacterial antigens

- Killed *Mycobacterium vaccae*. This is being tested as a prophylactic vaccine for the first time, in patients infected with HIV. It can drive regulatory T cells that downregulate Th2 in allergy models [402].

5) Boost CD4+ and CD8+ T-cell responses to the 65 kDa heat-shock protein (hsp65)

- DNA vaccine encoding hsp65. Induces CD4+ and CD8+ T-cell responses, and downregulates Th2-cell responses in animal models of TB [403].

1.7.5.2. Failure of BCG close to the equator

It has been known for decades that the efficacy of BCG vaccination varies in different parts of the world. In general it is least effective in developing countries where it is most needed [404]. There has been no universally accepted explanation for this

phenomenon. However, in these countries there is a background of Th2 response to some components of *M. tuberculosis*. This was clearly demonstrated in blood samples from Malawians in which PPD induced IL-5 secretion, whereas little IL-5 was seen in samples from the UK run in parallel (Dockrell H., personal communication). Worse still, BCG vaccination failed to down-regulate the IL-5 response (Dockrell H., personal communication). This Th2 activity might be attributable to the exposure of mother and child to helminths. BCG induced a Th2-biased response in babies that had been sensitised in utero to antigens of *Wuchereria bancrofti* or *Schistosoma haematobium*, because their mothers were suffering from these infections [405]. Presumably, contact with cross-reactive environmental mycobacteria would do the same thing in these babies. However, these organisms also induce a Th1 response in Malawi that is demonstrably protective [406], and the possible interplay between the Th1 and Th2 components is crucial.

Furthermore, there are discordant results of attempts to measure IL-4 in tuberculosis cases from developed and developing countries. Indeed, as already outlined in section 1.7.2 the levels of IL-4 detected in tuberculosis patients, or released by their lymphocytes when stimulated with mycobacterial antigen *in vitro*, differ markedly in different studies. At one extreme, in peri-equatorial countries, ELISA may detect free IL-4 easily in the patient's serum or in supernatants of patient's PBMCs cultured with tuberculosis antigen *in vitro*. At the other extreme, in the USA or Europe, IL-4 is not detected at all or only using sensitive quantitative RT-PCR, or by flow cytometry after non-specifically stimulating the cells. The method of IL-4 detection and geographical location is shown in figure 1.6, and a list of the relevant studies is shown in table 1.6. Clearly some of the patients studied in Europe or the USA will themselves have come initially from developing countries, but the distinction remains in the studies reviewed, perhaps reflecting that ongoing environmental exposure is important.

Table 1.6. IL-4 and method of detection (specified in the ‘assay method’ column) in patients with tuberculosis in developed and developing countries. In all cases peripheral mononuclear cells were used unless otherwise specified. One study using PBMCs from Indonesian subjects detected IL-4 after stimulation with PMA and ionomycin (serum IL-4 values are not given and it is unclear if stimulation with TB antigen was attempted [19]).

Abbreviated reference	Country	Assay method	Comment
Developed countries			
*Schauf V et al., 1993 [345].	USA	RT-PCR	Non-quantitative PCR using unstimulated cells IL-4 not detected in cell culture supernatants. Only serum evaluated.
*Barnes PF et al., 1993, [186].	USA	RT-PCR	
*Verbon A et al., 1999, [32].	Netherlands	Serum IL-4 not detect by ELISA	
*Seah GT et al., 2000, [17].	UK	Nested RT-PCR	Unstimulated cells.
*Surcel HM et al., 1994 [18].	UK	ELISPOT	Stimulation with TB antigen.
*Silveira H et al., 1997 [408].	Portugal	RT-PCR	IL-4 not detected in cell culture supernatants.
Developing countries			
*Jimenez-Martinez MC et al., 2004 [21].	Mexico	FACS- stimulation with TB antigen	ELISA not performed.
*Dlugovitzky D et al., 1999 [20].	Argentina	ELISA in serum	No cell stimulation studies performed.
*Montiel M et al., 2002 [22].	Venezuela	ELISA in serum	IL-4 also raised in PPD stimulated PBMCs.
*Sanchez FO et al., 1994 [23].	Columbia	ELISA– stimulation with PPD	n/a.
*Smith SM et al., 2002 [29].	The Gambia	FACS- stimulation with <i>M tb</i>	ELISA not performed.
*Bhattacharyya S et al., 1999 [347].	India	FACS- stimulation with <i>M tb</i>	IL-4 also detected by ELISA in supernatants.
*Sharma SK et al., 2002 [24].	India	ELISA	Serum IL-4 raised in miliary TB.
*Wang L et al., 2002 [346].	China	ELISA in serum	n/a.

Consequently, it has been suggested that vaccines should be screened in helminth-infected animals primed with environmental mycobacteria, rather than in immunologically naïve pathogen-free laboratory mice, which have few mature dendritic cells [342]. In such animals, the challenge dose can be adjusted to cause progressive disease in only a subset of the animals, so that an increase in the true percentage protection can be sought (rather than using bacterial load as a marker of vaccine efficacy where 100% of the animals have disease). Using such Th1/ Th2 primed animals, it might emerge that to provide protection in developing countries, it is more important to suppress subversive IL-4 production than to increase an already adequate Th1-cell response.

1.8. Conclusion

The data outlined in Chapter 1 provides an overview of the basic immunology relevant to TB, the prevailing putative mechanisms that may explain disease progression, the biological attributes of IL-4 and IL-4δ2 (effects, source, signalling pathways and regulatory mechanisms), the existing literature pertaining to IL-4 and TB, and how this pertains to vaccine design and the failure of BCG.

Nevertheless, there are many issues related to the IL-4 hypothesis that require clarification. From the data already reviewed, it seems clear that a sensitive and reliable quantitative assay is required to clarify the discordant findings of previous investigators. Moreover, it remains unclear whether IL-4 represents cause or is a consequence of disease in humans. Further information is required regarding IL-4 regulatory pathways, sources of IL-4 in human TB, compartment specific expression of human Th1 and Th2 cytokines (lung and blood), and the role of IL-4 and IL-4δ2 in HIV-TB co-infection. Before more detailed antigen fractionation, to identify the components that drive expression of Th2 cytokines is performed, the observation that TB antigen can preferentially drive IL-4 needs to be confirmed in cells from patients with TB. Data addressing these questions form the subject matter of this thesis.

CHAPTER 2. METHODS: OPTIMISATION AND VALIDATION OF A REAL-TIME RT-PCR ASSAY TO QUANTIFY IL-4 AND IL-482 GENE EXPRESSION.

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2.1. Introduction

Measuring human IL-4 in biological samples is challenging because of the biological properties of IL-4 and the insensitivity of existing immunoassays when using

unstimulated cells (discussed in detail in section 2.1.1. of this chapter). This necessitates the use of very sensitive techniques like reverse-transcription polymerase chain reaction (RT-PCR) to detect IL-4 in human samples. However, conventional RT-PCR has a number of drawbacks particularly if quantification is required (section 2.1.2.). Real-time RT-PCR addresses a number of these drawbacks (section 2.1.3. and 2.2.4.). However, despite these improvements there still certain inherent difficulties such as data normalisation, which requires the selection of a reference to control for errors during RT-PCR and for the amount of starting material (section 2.1.6). In addition to the above-mentioned considerations, this chapter outlines the development and performance characteristics of a probe based real-time RT-PCR assay to measure IL-4 and IL-4 δ 2 mRNA levels (section 2.3.1), measures undertaken to minimise measurement error, reference validation and the implications of the validation exercise (or lack thereof) on inter-group comparisons (2.3.2). The work outlining the development and validation of the assay has been published [409, 410].

2.1.1. Why PCR?

With the exception of subjects in tropical countries where IL-4 is markedly raised by the likely adjuvant activity of helminth infections, anyone who has worked with human biological samples will appreciate the challenge and difficulty of measuring IL-4. This is due to a number of technical factors related to the biological properties of IL-4; these are outlined in table 2.1. Failure to take many of these factors into account may result in discordant data and the generation of results that are sometimes of questionable biological significance [410]. For example Doherty and co-workers showed recently that detection of IL-4, even by RT-PCR, was substantially reduced within 2 hours in freestanding heparinised blood samples from Ethiopian patients; moreover, IL-4 was undetectable by ELISA in this study [411].

Table 2.1. Factors that make the measurement of human IL-4 technically difficult.

- i) The short half life of IL-4 mRNA in clinical samples; approximately 60 minutes [412]. IL-4 mRNA is no longer detected if heparinised blood samples are not processed within a couple of hours, as often occurs under field conditions [411].
- ii) The low mRNA copy numbers at which IL-4 is active; ~ a thousand fold below that of IFN- γ [413, 414]. Some publications fail to find IL-4 in patients or controls and so illogically conclude that it is not raised in the patients.
- iii) The binding of IL-4 and rapid internalisation to soluble or cellular receptors [415], which are widely distributed on cells in whole blood.
- iv) Pre-stimulation of cells by mitogens, used in some protocols is Th1 biased and causes potent induction of IFN- γ and rapid reciprocal down regulation of an already low copy number cytokine (IL-4) [35]. This effect is routinely studied by workers in the field of allergy, and adds further to measurement difficulties and misinterpretation of data in tuberculosis work.
- v) Immunoassays fail to distinguish between free IL-4 and that bound to sIL-4R [308] or to naturally occurring anti-IL-4 antibodies [311] (bound IL-4 is inactive).
- vi) Current immunoassays used by investigators, such as conventional ELISA, are not sensitive enough to detect biologically active amounts of IL-4, particularly in *unstimulated* cells, and even when blocking antibodies are used [34]. ELISPOT is more sensitive but still requires a pre-stimulation protocol [416]. Immunoassays using cytometric bead array [417] and flow cytometry are equivalent in sensitivity to ELISA [418]. There are no data evaluating the comparative sensitivity of the IL-4 cytokine secretion assay (Miltenyi Biotech), however a pre-stimulation protocol is usually required.
- vi) To overcome these problems investigators have used RT-PCR. However, failure to recognise the existence of a second cytokine, IL-4 δ 2, and use of primers which simultaneously amplify cDNA of IL-4 and its splice variant IL-4 δ 2 confuse most existing studies involving RT-PCR.

2.1.2. Conventional RT-PCR: limitations and drawbacks

In addition to the technical and sensitivity considerations outlined above there are no available immunoassays that specifically bind IL-4 δ 2. It remains unclear whether the current immunoassays measure IL-4, IL-4 δ 2 or both cytokines. Consequently, at the current time PCR remains the only alternative that simultaneously addresses the issues of assay sensitivity and specificity for IL-4 δ 2. Indeed, conventional nested RT-PCR used in earlier studies, the most appropriate technology available at the time, has been used to show that both IL-4 and the splice variant and antagonist, IL-4 δ 2, are upregulated in PBMCs from patients with active tuberculosis [17] and antigen stimulated mononuclear cells from healthy control subjects [26]. However, these studies suffer from the drawbacks inherent in conventional RT-PCR (table 2.2). Before these are discussed a few basic concepts integral to the understanding of PCR are discussed.

The early 1990's saw the widespread use of conventional RT-PCR, which was first described in 1987 [419]. Compared to other methods of RNA detection (in situ hybridisation, northern blot and RNAase protection assays) the sensitivity of RT-PCR was unrivalled. Two enzymatic steps are involved: the first is a retroviral enzyme (RNA-dependent DNA polymerase) based reverse transcription of RNA; the second is a heat stable DNA-polymerase dependent primer specific exponential amplification of cDNA using a thermal cycler, in a buffer containing deoxynucleotides and magnesium [420]. The kinetics of the PCR reaction is shown in figure 2.1 [421]. End point PCR discriminates poorly between different RNA input amounts because all reactions plateau at a similar end point (shown in figure 2.3 A). Moreover, the poor resolution of densitometry scanning, 2 logs at most [422], makes meaningful quantification challenging to say the least. Therefore, this simple yet sensitive assay could only provide qualitative (yes/ no) answers about gene expression.

However, requirements for diagnostic information (viral load, tumour diagnostics and study of gene expression in biological systems) demanded quantification of mRNA levels. Consequently, tedious and complicated protocols were developed to satisfy this need [423]. It was first necessary to undertake measurements at a cycle number in the exponential phase of the reaction. Quantification entailed using a single set of

primers to amplify the target sequence and a synthetic standard whose amplification product could be distinguished from the target sequence on an agarose gel [423]. For every sample identical aliquots of the template were co-amplified with a 'spike' (addition into the mixture) of the synthetic standard in different dilutions. Comparison of band intensities of the unknown amount of the target against a standard curve of the internal standard (competitor RNA or DNA) enabled quantification of the gene of interest. Numerous variations of this theme underpin all competitive RT-PCR protocols (competitive because reference and target sequences compete for the same primers) [39]. The protocol assumes equal amplification efficiencies of the target and synthetic standard, that the ratio of the target template and the standard remains constant and no heteroduplexes form between the target and the competitor. Furthermore, the technique relied on accurate quantification of the RNA reference standard by spectrophotometry (the difficulty with this approach is discussed in section 3.1.4). After 'quantification' the target copy number was normalised to an unvalidated reference (also called 'housekeeping gene' and discussed later) to control for the inter-sample input amount of RNA. However the semi-quantitative protocols that investigators were encumbered with in earlier studies, including a nested RT-PCR assay used to detect IL-4 in unstimulated cells [17, 26, 349], suffered from a number of drawbacks (table 2.2).

Table 2.2. Technical limitations and drawbacks of conventional RT-PCR quantitative protocols.

- i) Unreliable quantification and inconsistent results [424] due to the inherent nature of the technique and lack of standardisation. False positive rates can be as high as 28% [36] with error rates ranging from 10 to 60% [425].
- ii) This was compounded by the poor resolution and dynamic range of gel densitometry (2 logs at most) [422].
- iii) Highly variable reproducibility [425, 426] due to (i) and (ii) above.
- iii) Labour, time and reagent intensive thereby limiting the amount of samples that can be processed.
- iv) Prone to problems of contamination due to the need for post-PCR gel analysis.
- v) Almost routine failure to check the quality of RNA transcripts. Inadvertent use of poor quality transcripts result in erroneous data generation.
- vi) Difficulty in using predetermined amounts of RNA in cDNA reactions due to technical difficulty in quantifying RNA mass by spectrophotometry. This has been superseded by more accurate methods using micro fluidic technology [427].
- vii) Use of unvalidated internal reference genes leading to potentially erroneous results [37, 39]. The type of result generated may be influenced by the choice of reference gene used [326, 428].
- viii) Unequal efficiencies of the template and competitor RNA [429], hetroduplex formation between the template and competitor [426] and failure to maintain constant ratios between the target and competitor RNA.
- ix) Use of nested protocols (using the PCR product of one reaction in a subsequent PCR) compounded these problems

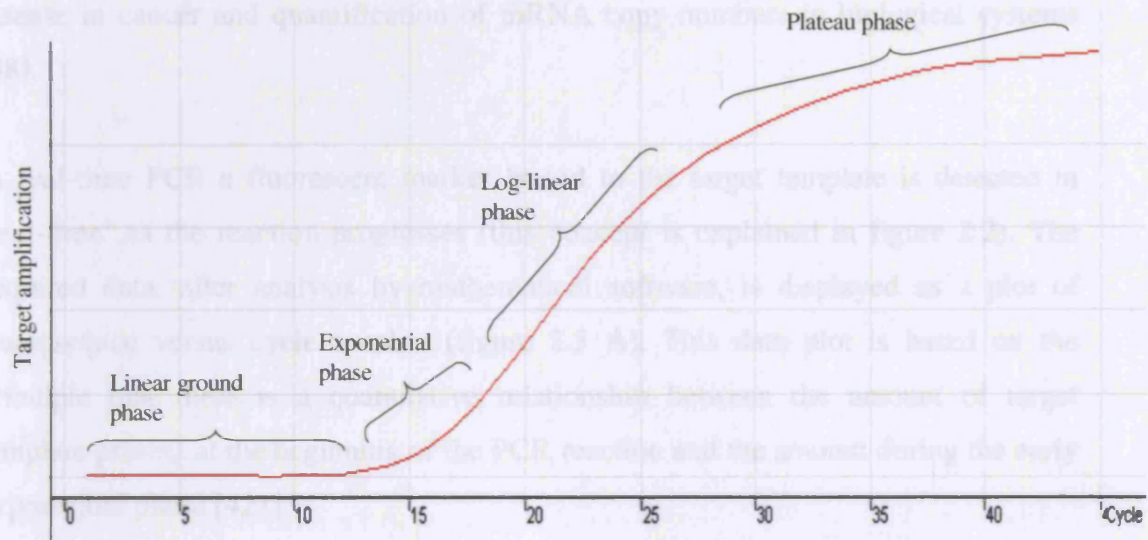


Figure 2.1. Kinetics of PCR amplification. During the first phase, the linear ground phase, there is relatively little increase in template. In the 2nd stage, the early exponential phase, the amount of amplified target directly proportional to the input amount of target RNA. During the 3rd phase the amplification efficiency is log-linear. The final plateau phase is characterised by a sharp drop in amplification due to reagent and substrate limitation; here there is little relationship between DNA input and amplified target. Consequently conventional RT-PCR has to be stopped in the exponential phase if quantification is attempted [421].

2.1.3. Real-time PCR: theory and general considerations

In the 21st century the difficulties that investigators faced, as outlined in the previous section, were dramatically circumvented by the advent of real-time RT-PCR. The major advantages of this technique are accurate and reliable quantification even at low copy numbers, high reproducibility, a wide dynamic range (10 logs), greater specificity because of template specific probes, and reduced contamination and workload because no post-PCR processing is required. Furthermore there are significant cost reductions due to multi-plexing and high throughput is attainable due to automation and simplification of the PCR technique [37-39, 430, 431]. Improvements in RNA assessment techniques and the recognition that the use of

unvalidated references to 'normalise' data could impact on study results, further improved the resolution and reproducibility of the technique [37, 38, 430]. Real-time PCR is now regarded as the gold standard for determination of viral load, residual disease in cancer and quantification of mRNA copy numbers in biological systems [38].

In real-time PCR a fluorescent marker bound to the target template is detected in 'real-time' as the reaction progresses (this concept is explained in figure 2.2). The acquired data, after analysis by mathematical software, is displayed as a plot of fluorescence versus cycle number (figure 2.3 A). This data plot is based on the principle that there is a quantitative relationship between the amount of target template present at the beginning of the PCR reaction and the amount during the early exponential phase [421].

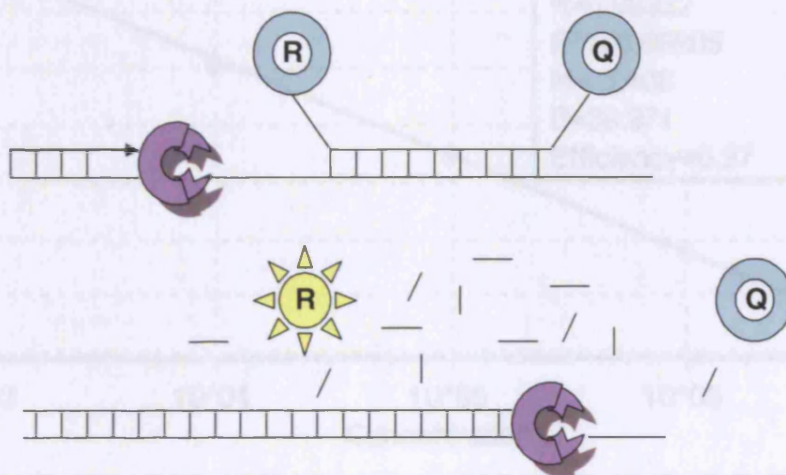


Figure 2.2. Detection chemistry: the hydrolysis probe. A probe (blocked primer that is labelled with reporter fluorophore (R) and a quencher (Q)) binds to a site on the target sequence downstream of the forward primer (arrowhead). Whilst closely associated, the excitation from the fluorophore is transferred to the quencher molecule without emission of a photon (this concept is called FRET (fluorescence resonance energy transfer)). During the PCR reaction, Taq DNA polymerase (purple semi-circle) digests the probe. As the reporter dye is no longer in close proximity to the quencher there is an increase in reporter emission intensity (yellow 'R'), which is easily detected [421]. There are number of detection chemistries (hybridisation probes, hairpin probes, scorpions etc) that are variations of the same theme. SYBR Green is DNA binding dye that emits fluorescence when generically bound to dsDNA; multiplexing is not possible with this dye.

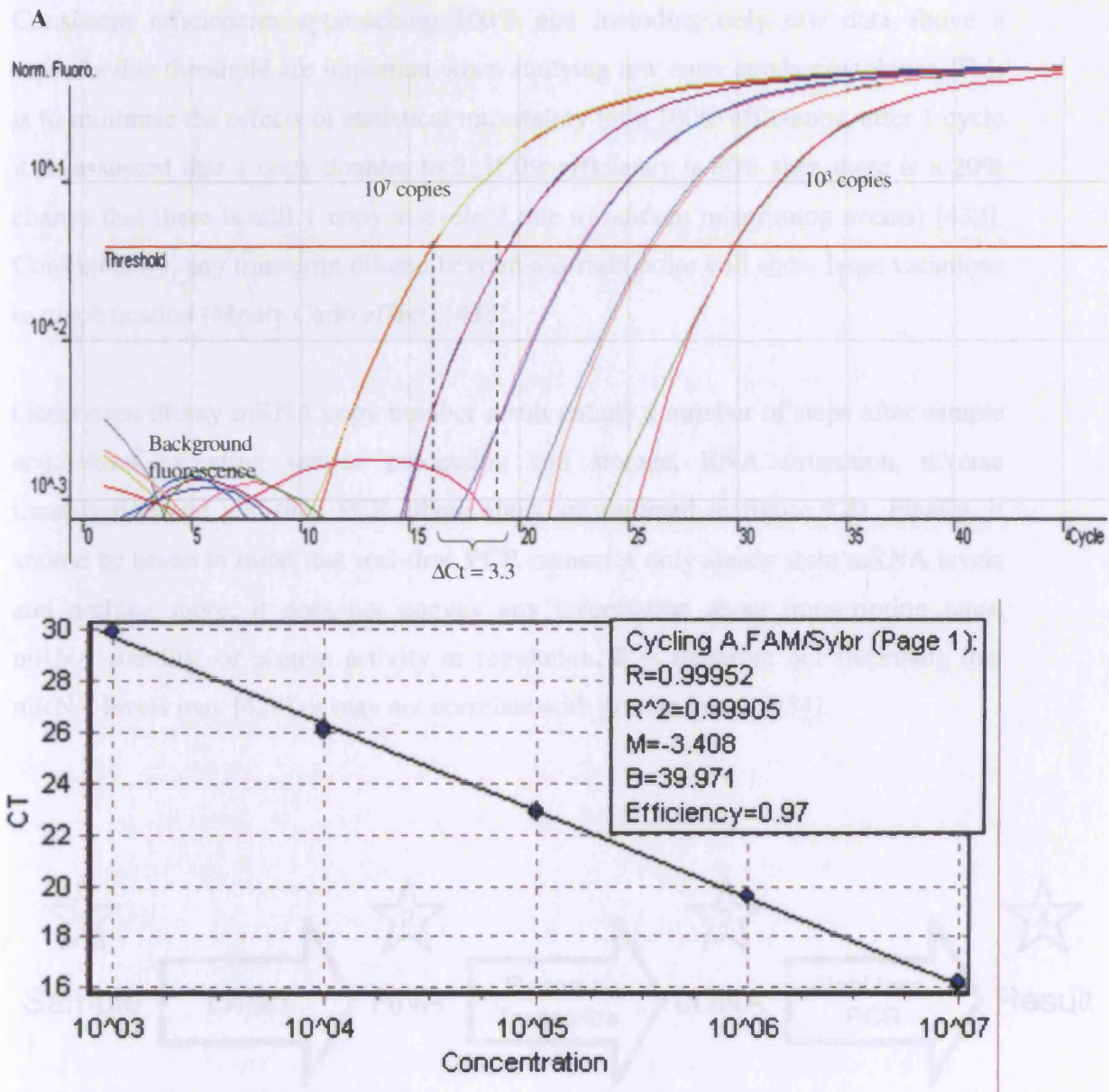


Figure 2.3 A and B. Standard curve (2.3 A) and calculation of amplification efficiency (2.3 B). The real-time PCR plot (shown in log scale) shows serial 10 fold dilutions of cDNA (2.3 A). The Ct (the cycle at which the fluorescence is statistically greater than the background; horizontal red line) is calculated in the early exponential phase. A slope is derived by plotting the Ct values against copy number (2.3 B). The efficiency (97% in this case) is calculated from the gradient of the slope (~ -3.3); implying that for every log increase in initial starting template the Ct occurs 3.33 cycles earlier (shown by vertical black lines). If the gradient of the slope was greater (~ -4.0) then the efficiency would be reduced to 78%; after 30 cycles this would result in an 80% reduction in the target copy number! The R^2 value reflects the accuracy of the dilution series and the pipetting repeatability of each data point [38, 430].

Consistent efficiencies approaching 100% and including only raw data above a reproducible threshold are important when studying low copy number cytokines. This is to minimise the effects of statistical uncertainty (at a 100% efficiency, after 1 cycle it is assumed that 1 copy doubles to 2; if the efficiency is 80% then there is a 20% chance that there is still 1 copy at cycle 2 due to random mispriming events) [432]. Consequently, any transcript diluted beyond a certain point will show large variations in amplification (Monte Carlo effect) [433].

Generation of any mRNA copy number result entails a number of steps after sample acquisition including sample processing and storage, RNA extraction, reverse transcription and real-time PCR (these steps are outlined in figure 2.4). Finally, it should be borne in mind that real-time PCR measures only steady state mRNA levels and nothing more; it does not convey any information about transcription rates, mRNA stability, or protein activity or regulation. It is therefore not surprising that mRNA levels may [428] or may not correlate with protein levels [434].

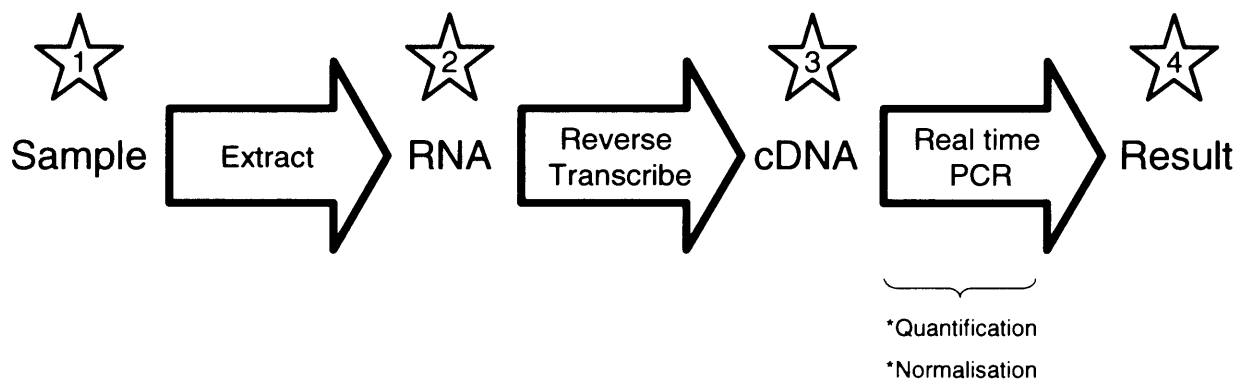


Figure 2.4. Sequential steps that are required for the generation of mRNA copy numbers. Quality control and maintaining RNA integrity has to occur during every step prior to RT. Particularly important is the prevention of gene induction or RNA denaturation at step 1 and checking RNA quality at step 2; these steps are often neglected and their details omitted, in the published literature. Prior to the acquisition of a final result the raw real-time PCR data must be quantified and then normalised to a reference to control for the amount of starting material and errors during steps 1 to 4.

2.1.4. Sample processing, RNA extraction, RNA quantification

As often stated: 'garbage in garbage out'! The generation of biologically meaningful and accurate gene expression data are heavily dependent on the input quality of the mRNA template. This is influenced by sample processing, RNA extraction and suitable nucleic acid storage. It is remarkable that most published literature makes no mention of whether RNA quality was checked, let alone how it was done. Guidelines, which will soon make it mandatory for investigators to report on the method of sample stabilisation, RNA quantification and RNA quality assessment, are being developed.

Once RNA has been extracted its integrity must be ascertained; denatured templates should be discarded. The traditional method was to check the clarity of the 18s and 28s band intensity on a formaldehyde agarose gel [435]. However the resolution is crude and the requirement for large amounts of RNA make it impractical for most investigators. This technique has now been superseded by the Agilent 2100 Bioanalyser using the RNA 6000 Labchip[®] (see figure 3.2). This benchtop instrument uses a combination of microfluidic and capillary electrophoresis technology to simultaneously analyse RNA mass and quality over a wide dynamic range [435, 436]. The advantages are simultaneous assessment of RNA quality and quantity, and the requirements for very small amounts of RNA. DNA size and concentration can also be assessed using a different chip. Alternative methods to assess RNA integrity include signal strength determination of four preselected mRNA transcripts [435] and the ratio of mRNA fragments proximal and distal to the 3' end of a transcript [437].

Precise RNA quantification is important as it allows efficient use of limited amounts of RNA, is crucial if normalisation to total RNA is used and facilitates accurate data normalisation, irrespective of the method of normalisation used (discussed later).

2.1.5. Reverse transcription

Reverse transcription (RT) entails converting the mRNA transcript to a single stranded complementary copy of cDNA using a reverse transcriptase enzyme, which is a RNA-dependent DNA polymerase derived from mutagenised avian (AMV) or murine (MMLV) tumour viruses [37, 39]. The enzyme adds deoxyribonucleoside

triphosphates to a growing DNA chain within a buffer containing divalent ions (Mg^{2+} and Mn^{2+}), primers and RNAase inhibitors. Activity of the enzyme requires binding of a primer to the RNA molecule to initiate DNA synthesis. This may be a gene specific primer, a random hexamer or oligo-dT (poly-T oligonucleotides), which anneals to the 3' poly-A tail of mRNA. There is ongoing debate about which primer type is most efficient though random hexamers are known to overestimate copy numbers by up to 19 fold [438]. RT is one of the most variable steps in the multistage RT-PCR process. The variability is related to the type of priming method used and carryovers from the RNA extraction step (phenol, alcohol, salts), which can modulate RT efficiency [439]. Control for variation during this step is discussed under normalisation (section 2.1.6.2).

2.1.6. Real-time PCR data processing

Acquiring the real-time PCR data is probably the most straightforward part of the multi-step RT-PCR exercise (figure 2.4). However, the raw data must be quantified (relative or absolute) and then normalised to control for varying amounts of starting material and errors during the PCR process.

2.1.6.1. Quantification (absolute versus relative)

The copy number in the sample of interest can be expressed in relation to a standard curve containing known numbers of nucleic acid copies (absolute quantification) or relative to a control sample (also known as a calibrator sample; here results are expressed as a ratio of the sample of interest relative to the calibrator). Unlike relative quantification where the patient and control samples have to be run on the same plate (methods reviewed in [440-442]), absolute quantification is preferable when large numbers of samples need to be assayed over time or at different study sites [421]. Because the same dilution series is co-amplified on every plate this method also facilitates the monitoring of inter-plate efficiency, day-to-day reproducibility and exclusion of results below a detection threshold (lowest dilution series).

2.1.6.2. Normalisation and Data interpretation

To optimally realise the advantages of real-time PCR and produce meaningful results good normalisation strategies are required to control for experimental error introduced during the multistage process (inter-sample variability of RNA extraction, RT and pipetting error; figure 2.4), and to control for differing amounts of starting material (input RNA). It is worth mentioning at this point that operator error is an important source of variability [37] and precision pipetting, regular pipette calibration and use of robotic technology (if resources permit) is crucial. The concept of normalisation is outlined in figure 2.5. There are many strategies that can be chosen; these include normalisation to sample size, total RNA, an internal reference (also called a housekeeping-gene), genomic DNA or an 'alien' molecule that is introduced during the RNA extraction stage (reviewed in [436]). Combining then first 3 strategies are additive in reducing measurement error and will produce the most robust RT-PCR data [436].

Normalising to a reference gene is a simple and popular method for internally controlling for error in real time RT-PCR. This strategy targets RNAs encoded by genes, which have been collectively called house keeping genes and is advantageous as all the steps required to obtain the final PCR measurement (figure 2.4) are controlled for [436]. Both the gene of interest and the reference gene are measured using real time RT-PCR. Commonly used house keeping genes like β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA were used as references for a number of years before the advent of real time RT-PCR. Their use was acceptable for non/semi-quantitative techniques like northern blot, RNase protection assays and conventional RT-PCR where a qualitative change was being measured. However, because of ease and convenience, they continued to be used for quantitative real-time PCR. In the last 5 years it became apparent that reference genes like GAPDH and β -actin were highly variable under different experimental conditions and were unsuitable for use as 'one size fits all' reference genes. This consideration is discussed in more detail later.

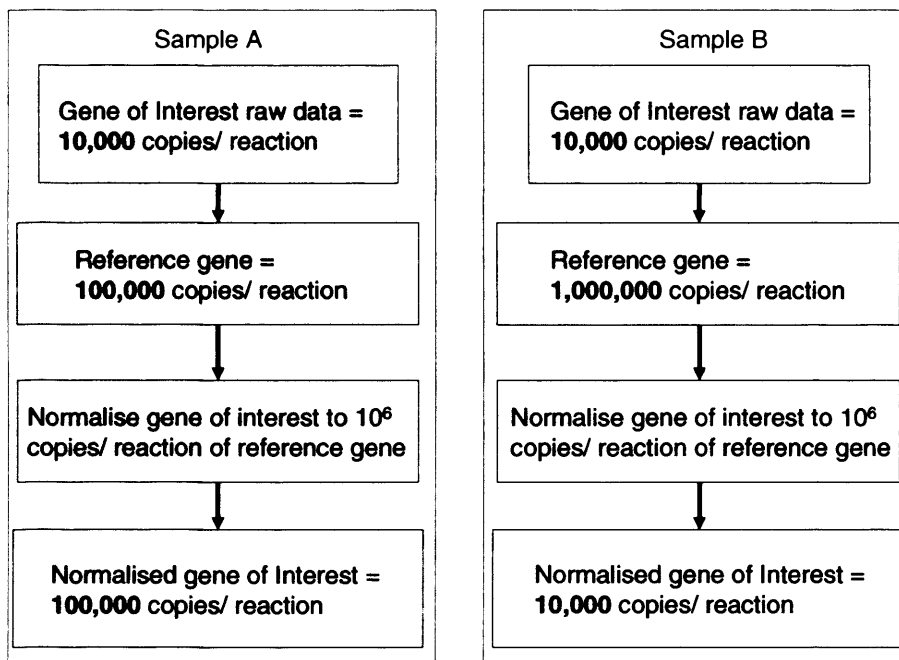


Figure 2.5. The concept of normalisation. In reality sample A (tissue biopsy) has greater quantities of the target gene of interest than sample B. However, the raw PCR data reveals that sample A and B have similar amounts of transcripts. After correcting for the amount of starting material (sample A had $1/10^{\text{th}}$ of the mass of sample B), it emerges that sample A actually has 10 times more transcripts of the target gene than sample B per unit/ mass. The use of the internal reference also corrects for pipetting error, and variability in the RNA extraction and RT steps. However, in reality there can be considerable variability of the reference, and therefore, validation is required.

Some have argued that the above-mentioned factors are irrelevant and the overall study conclusions will be unaffected because reference gene variability is likely to be equally distributed in the intervention and control group [443]. Of particular note are the findings of Bas *et al* [444] and Tricarico *et al* [428] demonstrating that use of unvalidated reference genes may impact upon study results. Nevertheless, whether the use of unvalidated reference genes can alter statistically significant inter-group differences remains unproven. The degree of reference gene variation that is acceptable also remains contentious. Both these issues are discussed later in relation to the data presented in this chapter.

2.2. Methods: development and validation of a real time RT-PCR assay

Assay development and validation in the methods (section 2.2 of this chapter) is presented in two parts. The first part describes methods relevant to the selection of a suitable reference gene. The ABI Prism 7000 real-time PCR machine was used for these experiments. The second part outlines the impact on study conclusions, of using a validated versus unvalidated reference, in real-time PCR experiments. The Rotorgene (Corbett Research) real-time PCR system was used for the latter and subsequent experiments described in this thesis.

2.2.1. Selection of optimal reference gene

2.2.1.1. Patients and samples

Clinical samples (n = 28) were taken from four patients with smear-positive pulmonary TB (PTB), and from four healthy individuals, and the cells harvested from PBMC cultures of four additional patients with PTB. In order to maximize inter-individual variability, we chose subjects of different ages (from 26 to 50 years), sex, and ethnicity (Caucasian, Somalian, Indian, Chinese, Filipino, and Black African). Informed consent was obtained from patients, and the relevant hospital ethics committees approved the study.

Whole blood (20 mL) was taken, and 2.5 mL was immediately transferred into PreAnalytiX PAXgene™ blood RNA tubes (Qiagen, Valencia, CA, USA) to fix the mRNA expression profile [445]. The remaining blood was transferred to a heparinized container and transported in a thermo flask at 37°C. To assess the effect of delayed sample processing on the reference, heparinized blood was incubated at 37°C for a further 4 hours on reaching the laboratory, after which a further 2.5 mL sample was transferred to a PAXgene tube.

Blood taken from four of the patients with PTB was layered over a Ficoll-Paque® gradient (Amersham Biosciences, Piscataway, NJ, USA) and the PBMCs isolated. PBMCs were cultured in triplicate in medium at a cell concentration of 1×10^6 cells/mL (final volume of 0.5 mL) in 24-well plates. Cells stimulated with TB antigen

and harvested at baseline and days 2/3. Days 4/5 were used for housekeeping gene expression studies. Control wells were challenged with phytohemagglutinin, *Mycobacterium vaccae* sonicate, or no antigen. Cell viability was assessed at each harvest with trypan blue, and a proliferation assay was performed on days 4/5 to quantify the proliferative response to antigen challenge. Further details of culture conditions are described in chapter 3.

2.2.1.2. Selection of house keeping genes

To begin with, the gene expression levels of 10 housekeeping genes (designated group 1) were studied using a commercially available assay (TaqMan[®] human endogenous control plate; Applied Biosystems, Foster City, CA, USA) in the blood and culture samples [428]. The 10 genes investigated in this assay are shown in table 2.3. Group 2 genes were selected as they were among 47 out of 535 maintenance genes, found by microarray data to be relatively stable, in 11 different human tissues [446] and since they were found to be invariant in human CD4 T cell cultures from cord blood [447]. The aim was to identify a housekeeping gene with minimal variability under our different experimental conditions. As some of the genes of interest are low-copy number cytokines, it was anticipated that a significant change between study groups was likely to be small. Therefore, a standard deviation of less than 2-fold from the mean expression level of the gene was chosen as a requirement for suitability as a reference gene. Ribosomal subunit RNAs were not used as housekeeping genes, as oligo(dT) was used as a primer for cDNA synthesis, and compared to specific downstream primers, random hexamer primers have been shown to overestimate mRNA copy numbers by up to 19-fold [438].

Table 2.3. Housekeeping genes selected for group 1 and group 2 panels.

GROUP 1 HOUSEKEEPING GENES	GROUP 2 HOUSEKEEPING GENES
Human acidic ribosomal protein (HuPO)	Human acidic ribosomal protein (HuPO)
β -Actin (BA)	Elongation Factor-1-alpha (EF-1- α)
Cyclophilin (CYC)	MLN51
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Ubiquitin conjugating enzyme (UbcH5B)
Phosphoglycerokinase (PGK)	
β_2 -Microglobulin (B2M)	
β -Glucuronidase (GUS)	
Hypoxanthine ribosyl transferase (HPRT)	
Transcription factor IID TATA binding protein (TBP)	
Transferrin receptor (TfR).	

2.2.1.3. Isolation of RNA extraction and RT

RNA was isolated from whole blood collected in PAXgene tubes using the PreAnalytiX PAXgene blood RNA kit (Qiagen) and from harvested PBMCs using the RNeasy[®] Mini kit (Qiagen). All samples were DNase (Qiagen) treated and the standard precautions of RNA handling and storage were observed. The RNA template was qualitatively assessed and quantified using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano Labchip[®] kit for blood-derived RNA and the RNA 6000 Pico Labchip kit for culture-derived RNA (all from Agilent Technologies, Palo Alto, CA, USA). For illustrative purposes examples of Bioanalyser tracings are shown in chapter 3, figure 3.2. Total RNA extraction varied from approximately 1–5 μ g for blood and 0.1–0.8 μ g from 5×10^5 cells. To study the effect on housekeeping gene expression, we used a fixed amount of input RNA for each cDNA reaction. Limited RNA quantities dictated input RNA amounts to be 600 ng for PTB patients, 400 ng for healthy volunteers, and 3 ng for the PBMC culture reactions. Reverse transcription reactions were performed following the manufacturer's instructions using Omniscript[®] Reverse Transcriptase (Qiagen) for blood-derived RNA and Sensiscript[®] Reverse Transcriptase (Qiagen) for culture-derived RNA in 60- μ L reactions.

2.2.1.4. Real-time PCR

The PCRs for group 1 genes were performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). In each reaction, approximately 15 and 0.1 ng of reverse-transcribed RNA (based on the initial RNA concentration) was used for blood and cell culture PCRs, respectively. The TaqMan endogenous control plate assay was used according to the manufacturer's instructions, with the exception of the 18S ribosomal RNA reaction, which was omitted (initial step of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, in a 50- μ L reaction volume). Using these parameters, the reaction efficiency approaches 100%. The primer and probe sequences for group 2 were obtained from previously published work [447]. These sequences are shown in Table 2.4. Primers and probes were synthesized by MWG (Ebersburg, Germany) and Sigma-Genosys Ltd. (Cambridgeshire, UK), respectively, with the exception of human acidic ribosomal protein (HuPO), which for this particular experiment was purchased from Applied Biosystems (Assays-on-Demand™). Primers and probes were used at 500 and 300 nM, respectively, in a 50- μ L reaction. The reactions were performed on the ABI 7700 Sequence Detection System with the same parameters as the group 1 genes.

Reaction efficiencies for group 2 genes (range of 96%–100%) were derived from standard curves using serial dilutions of purified PCR product. Amplification of the correct product was confirmed by using the Agilent 2100 Bioanalyzer (with the DNA 500 Labchip Kit; Agilent Technologies) [448]. All reactions were run in duplicate, and cycle threshold (Ct) values for group 1 genes were normalized to the internal positive control (IPC) to control for interplate variability. HuPO measurements were used to compare the results between groups 1 and 2. Non-template controls were used as recommended [39].

Table 2.4. Primer and probe sequences used to quantify gene expression by real-time PCR.

All gene abbreviations are defined in table 2.3.

Genes	* Probe sequence 5'-(FAM- TAMRA)-3' ◇ primer 1 -5'-3' □ primer 2 -5'-3'	Product size	R ²
EF-1- α	* AGCGCCGGCTATG CCCCTG ◇ CTGAACCATCCAGGCCAAAT □ GCCGTGTGGCAATCCAAT	59bp	0.992
MLN51	* AGGCCTGTGGAAGCTGGTGGGC ◇ CAAGGAAG GTCGTGCTGGTT □ ACCAGACCGGCCACCAT	72bp	0.999
UbcH5B	* TGATCTGGCACGGGACCC TCCA ◇ TG AAGAGAATCCACAAGGAATTGA □ CA ACAGGACCTGCTGAACACTG	64bp	0.999
HuPO	Applied Biosystems® (Assays-on-demand™)	110bp	0.999
Group 1	Taqman Human Endogenous Control Plate, Applied Biosystems®	n/a	n/a

2.2.2. Effect of reference gene selection on inter-group comparisons

This section describes the methods relating to the second set of experiments, which outline the effect of using an unvalidated reference gene to normalise real-time PCR data.

2.2.2.1. Patients and samples

For this part of the study, 21 patients with proven PTB and 21 matched healthy volunteers were recruited. Further details regarding diagnosis and clinical characteristics are described in chapter 4.

Whole blood (2.5ml) was immediately transferred into PAXgene Blood RNA tubes (PreAnalytix, Qiagen), and total RNA was extracted and reverse transcribed as already described (20 µL reaction, Omniscript, Qiagen). RNA integrity and quantity was assessed using an Agilent Technologies 2100 bioanalyzer. In each cDNA reaction 150ng of input RNA was used. Levels of mRNAs encoding IL-4 were measured in whole blood from all patients at baseline (before or within the first 2 weeks of anti-TB treatment) and in 10 patients at approximately 6 months after initiating anti-TB treatment. Design and optimisation of HuPO and IL-4 primers and probes are described in chapter 3 (General methods).

2.2.2.2. Real-time PCR & data analysis

The PCR reactions were performed using the Rotorgene (Corbett Research). In each reaction, ~15 ng of reverse transcribed RNA (based on the initial RNA concentration) were used for reactions containing whole blood. Reaction parameters and primer sequences for IL-4 mRNA and HuPO are described in detail in chapter 3. All reactions were run in duplicate and nontemplate controls were used as recommended [39].

Cytokine mRNA values from TB samples were normalized to a conventional reference gene (GAPDH) or (HuPO), a validated reference gene. The use of HuPO was validated, as outlined in the section 2.2.1 above, by assessing the variability of 13 reference genes in samples (blood and mononuclear cell cultures) from patients with tuberculosis and from healthy volunteers [409].

For the purposes of data analysis, raw data replicates that were nonreproducible and below the detection limit of the assay (10 copies) were given an arbitrary value of 1 copy in the final analysis. Results were analyzed using the Mann–Whitney U test, the Wilcoxon matched-pairs test, and the Student's t test.

2.3. Results

2.3.1. Reference gene validation

The results presented in section 2.3.1 pertain to finding a suitable reference for normalising real-time PCR data in donors with TB and healthy volunteers using different biological samples.

2.3.1.1. HK gene expression in whole blood

The median expression level (Ct value) of group 1 and group 2 HK-genes for whole blood (n = 16) are shown in figures 2.6 A and B. Also shown are Ct (25th and 75th percentile) values, and ranges for each house keeping gene. Figure 2.7 shows the standard deviation (SD) expressed as a fold change from the mean and range expressed as maximum variability for selected house keeping genes in whole blood.

The most stable HK gene in whole blood was HuPO with an average fold change of < 2 and a maximal variability of < 5 fold. There was considerably greater variability for GAPDH, β -Actin and HPRT (maximum variability of 20-25 fold). When gene expression at 4 hours was compared to baseline there was little difference in the expression levels of all 13 genes (average FC for all genes over 4 hours = $1.37 \pm 0.33x$).

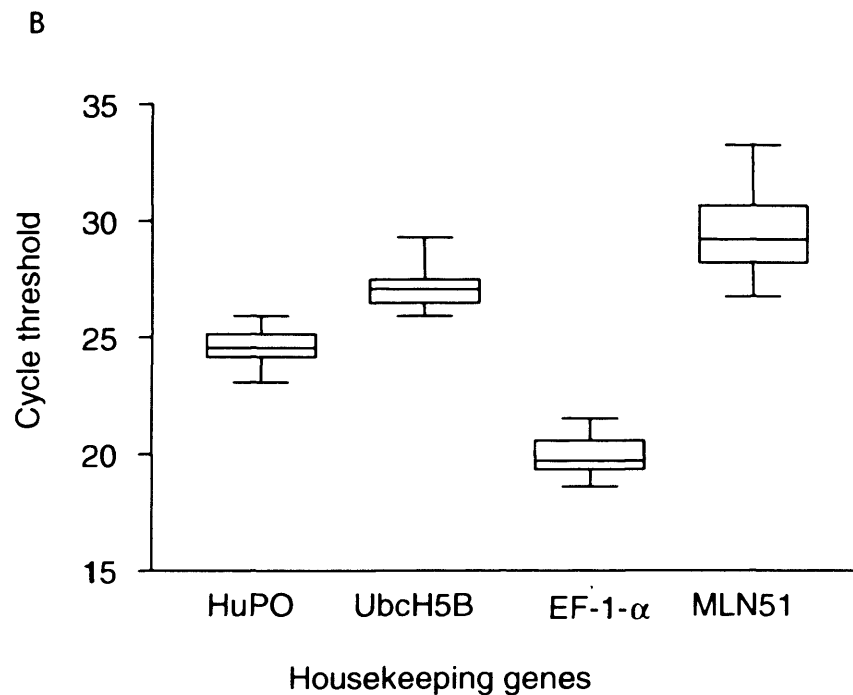
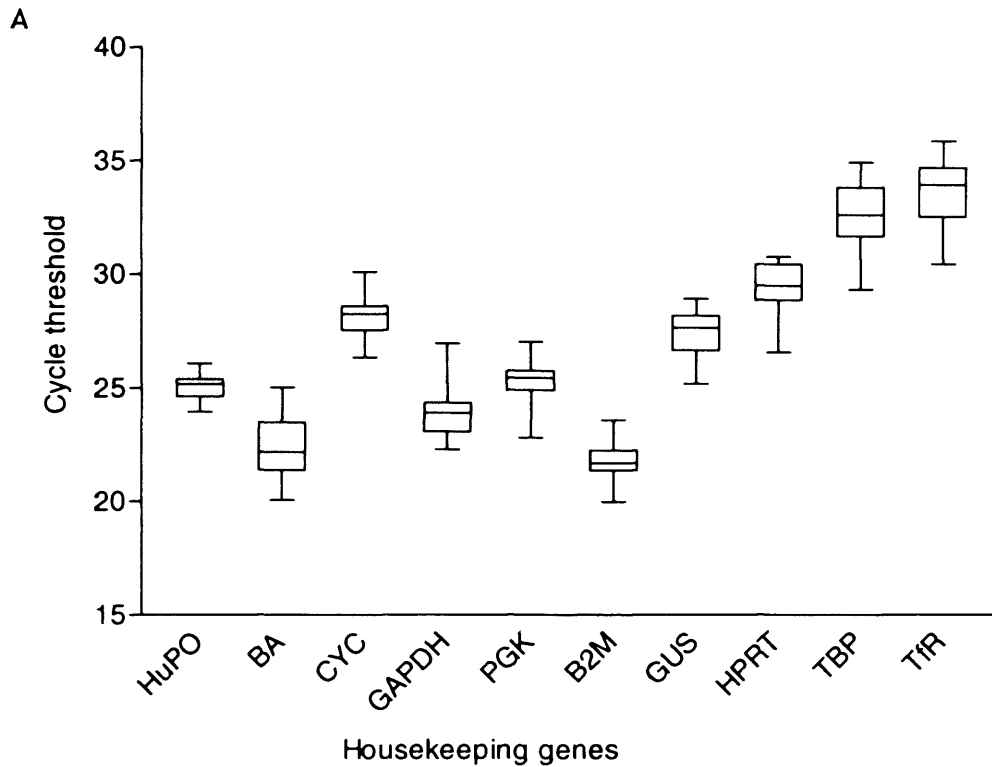


Figure 2.6 A and B. Real-time PCR cycle threshold values. Expression levels of Group 1 (A) and 2 genes (B) are shown as medians (lines), 25th percentile to the 75th percentile (boxes) and ranges (whiskers) for 16 human blood samples (4 normal and 4 tuberculosis patients at two time points 4 hours apart). All gene abbreviations are defined in table 2.3.

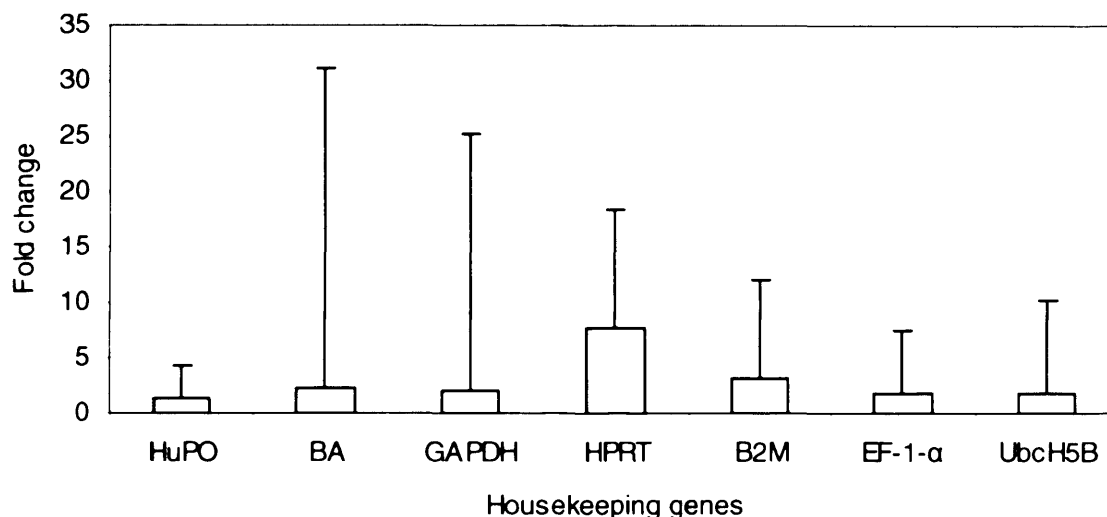
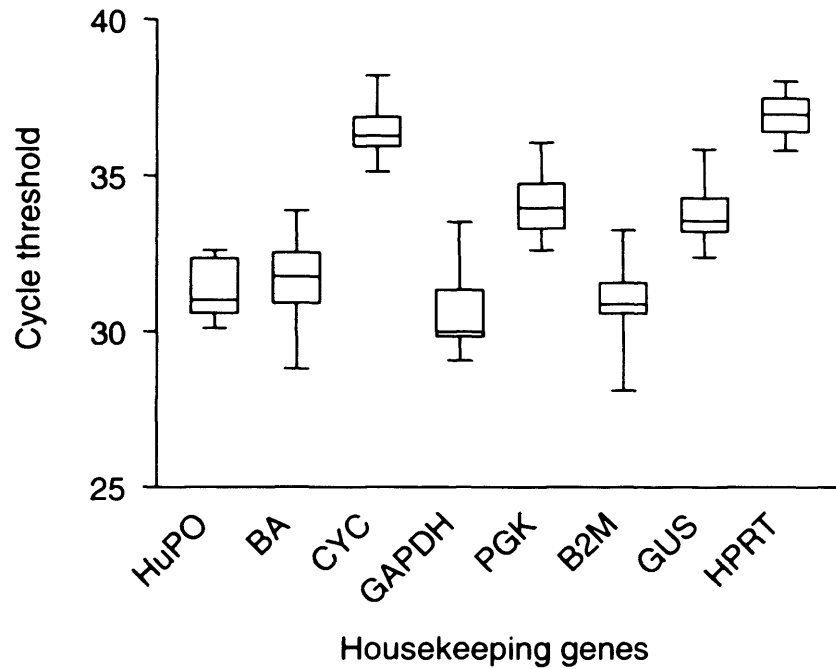


Figure 2.7. Fold change in gene expression. Variability of selected group 1 and 2 genes in human whole blood shown as an average fold change from the mean (columns) and maximum fold change (error bars). All gene abbreviations are defined in table 2.3.

2.3.1.2. HK gene expression in PBMC culture

The median expression levels (Ct value) of group 1 and group 2 HK-genes for PBMC culture (n = 12) are shown in figures 2.8 A and B. Figure 2.9 shows the Ct values expressed as fold changes. The most stable HK genes in PBMC culture were HuPO and HPRT with an average fold change of < 2 and a maximal variability of ~5 fold each. The most variable genes were GAPDH, β 2 microglobulin, β Actin and EF-1- α with an average fold of >2 and a maximum variability of 20-35 fold.

A



B

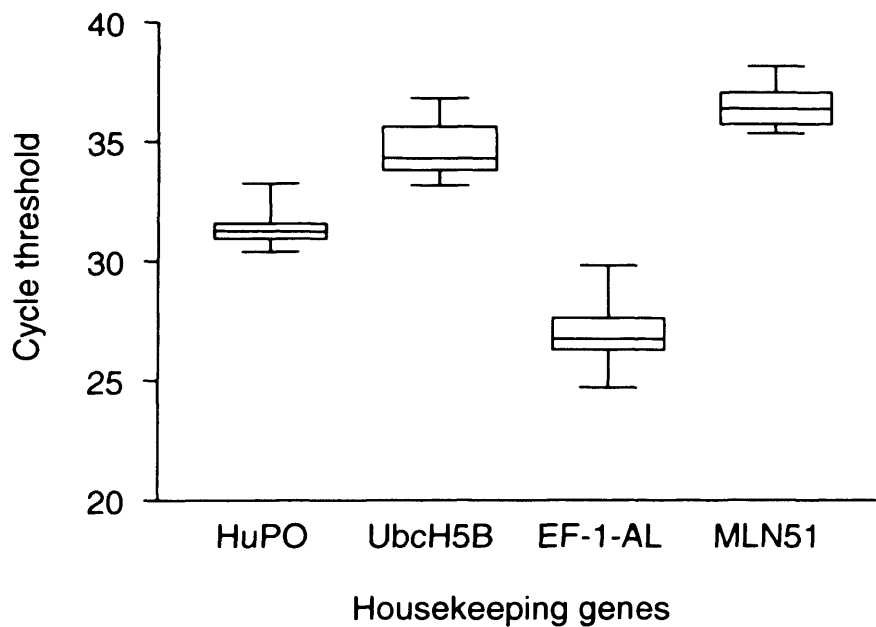


Figure 2.8 A and B. Real-time PCR cycle threshold values. Expression levels of Group 1 (A) and 2 genes (B) are shown as medians (lines), 25th percentile to the 75th percentile (boxes) and ranges (whiskers) for 12 human PBMC culture samples (4 tuberculosis patients at 3 time points). All gene abbreviations are defined in table 2.3.

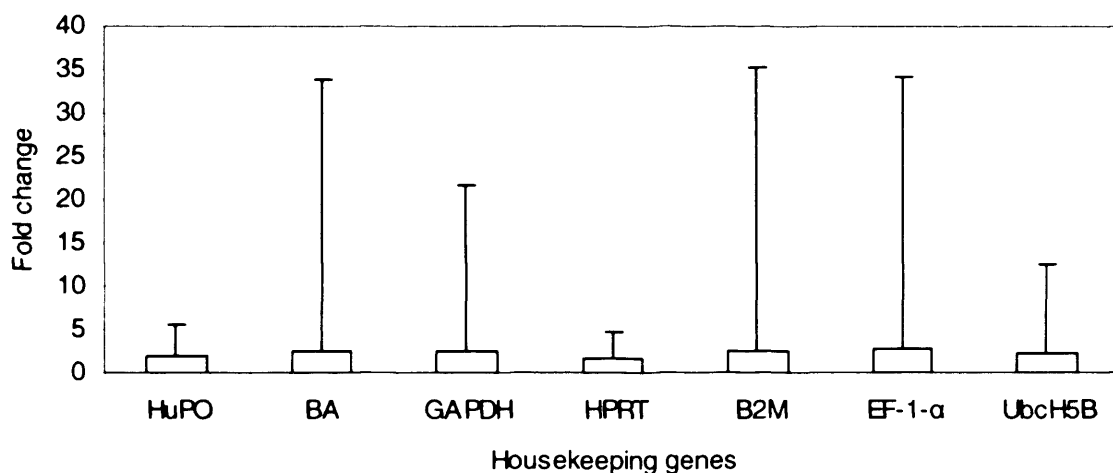


Figure 2.9. Fold change in gene expression. Variability of selected group 1 and 2 genes in human PBMC culture shown as an average fold change from the mean (columns) and maximum fold change (error bars). All gene abbreviations are defined in table 2.3.

2.3.2. Effect of reference genes on inter-group comparisons

The data presented above demonstrate that many conventionally used reference genes vary widely in mRNA expression and only one gene (HuPO) was found to be suitable as a reference. The data presented in the following section demonstrate the effects of using a validated (HuPO) versus un-validated reference (GAPDH) on the target gene expression.

2.3.2.1. Normalisation of data with HuPO vs GAPDH in whole blood.

An increase in expression of IL-4 in tuberculosis patients can be masked when real time RT-PCR is used with an inappropriate reference gene (figure 2.10 A and B). Data normalised to HuPO resulted in a highly significant, $p = <0.0001$, ~ 7 fold increase in IL-4 mRNA expression in TB, which was abrogated when the data were normalised using GAPDH.

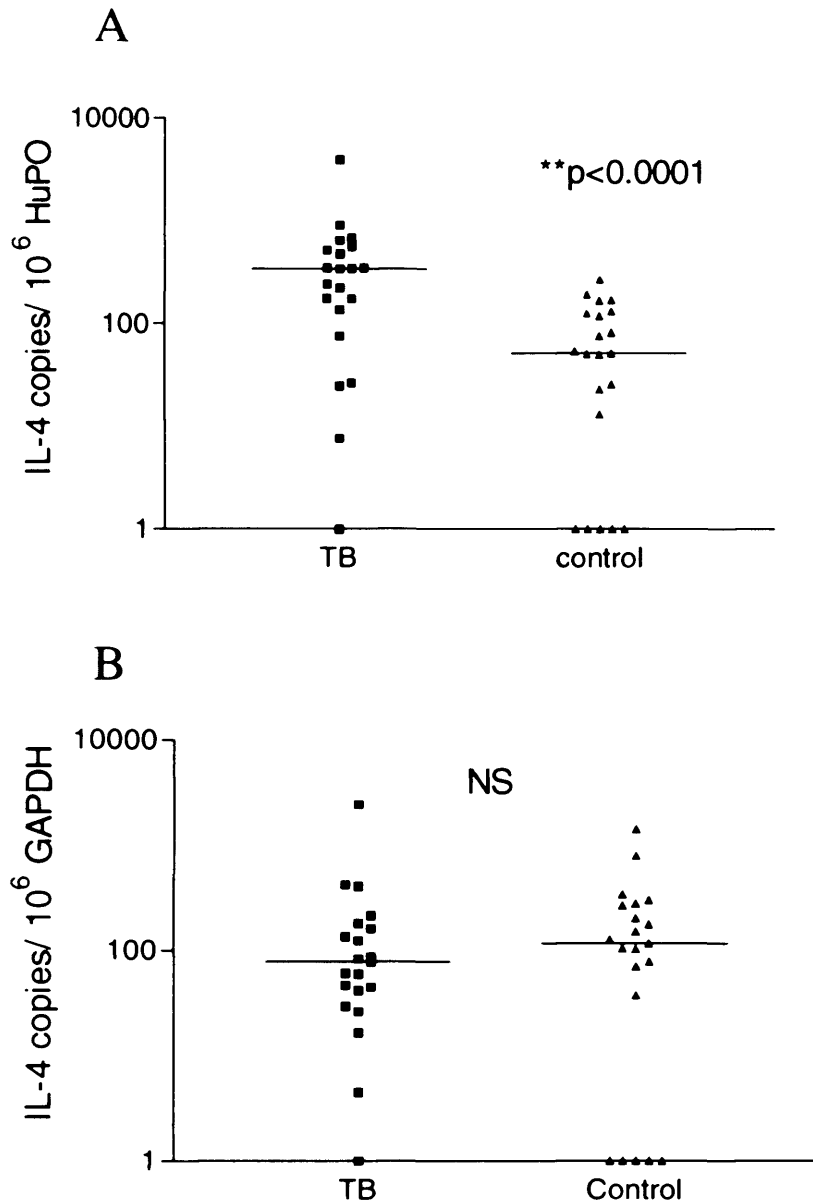


Figure 2.10 A and B. Differences in IL-4 mRNA expression in patients with pulmonary tuberculosis and healthy controls. Inter-group gene expression differences were statistically significant when data was normalized to HuPO but not GAPDH. Raw data is normalised to ~106 copies of HuPO (A) or ~106 copies GAPDH (B).

2.3.2.2. Comparison of data between time-points using different reference genes.

Normalisation of IL-4 gene expression with HuPO suggests that anti-TB treatment results in a non-significant decrease in IL-4 mRNA expression. However, if GAPDH is used a ~5 fold significant increase is observed (figure 2.11 A and B).

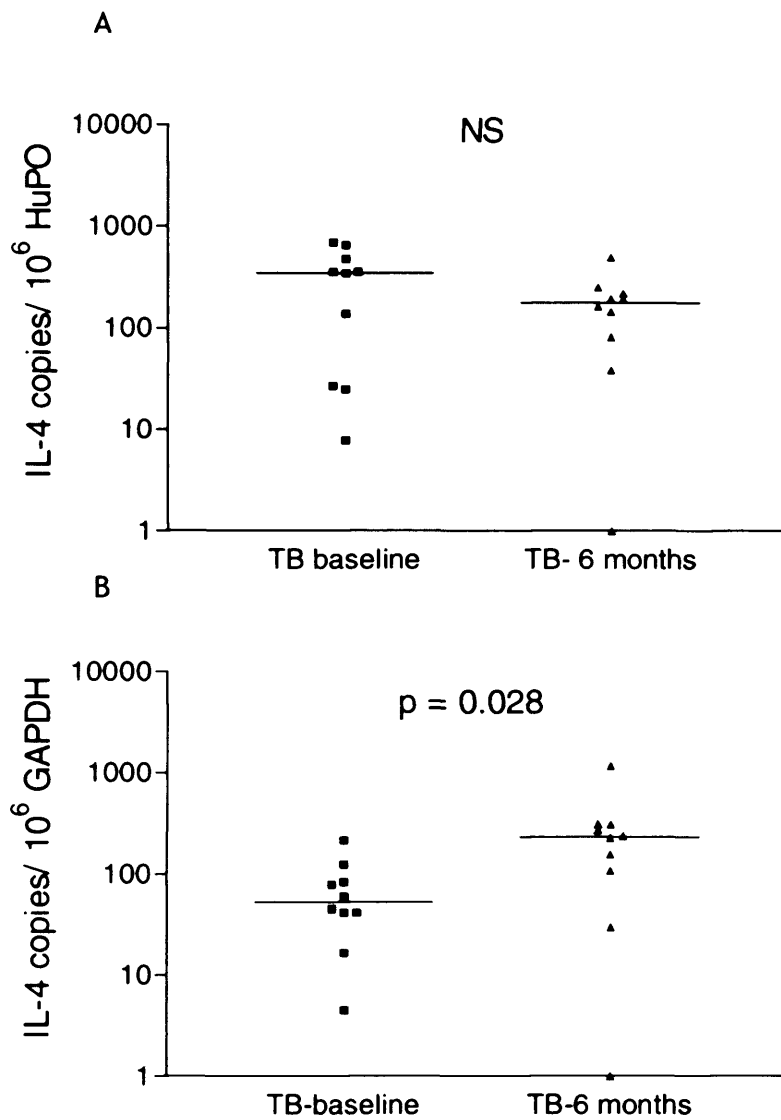


Figure 2.11 A and B. Differences in IL-4 mRNA expression in untreated patients with pulmonary tuberculosis and patients treated for 6 months. Inter-group gene expression differences were statistically significant when data was normalized to GAPDH but not HuPO. Raw data is normalised to $\sim 10^6$ copies of HuPO (A) or $\sim 10^6$ copies GAPDH (B).

2.4. Discussion

2.4.1. Reference gene validation

Though a number of previous studies [413, 449, 450] have assessed the experiment-specific suitability of reference genes, none have screened genes suitable for normalisation when using human biological samples derived from patients with active

tuberculosis. A study of the expression levels of 13 HK genes in patients with tuberculosis revealed only one gene suitable for normalisation of RNA levels. According to the selection criteria of less than two fold, HuPO was the most suitable gene overall (blood and PBMC culture). GAPDH and β -Actin did not satisfy the suitability criteria and had an unacceptably high maximal variability. Moreover, genes found to be invariant in mitogen stimulated human T cell cultures [447], were found to be unsuitable when studied in human PBMC cultures stimulated with TB antigen. Other genes like HPRT were more variable in whole blood than in proliferating PBMC cultures. These data show that HK genes are highly specific for a particular experimental model and validation for each situation, on an individual basis is a crucial requirement.

2.4.1.1. Reference genes and choice of a suitable normalisation strategy

Logically, if the reference or 'house-keeping gene' is to be used to compensate for errors and the amount of starting material it should not theoretically vary between individuals, different tissues and under different experimental conditions. However, in reality there is considerable inter-individual and inter-tissue variability in reference gene expression [37, 421, 430, 436]. Considerable changes in levels were noted with viral infection (18s RNA), tissue type (HPRT) and cell type (β -actin) [436]. More recently there have been a number of reports that demonstrate that the classic reference genes can vary considerably and are unsuitable for normalisation purposes due to large measurement error [37, 39, 409, 421, 428, 430, 431, 436]. Using rRNA (28S and 18S) are also problematic as they can remain intact despite mRNA degradation, they are transcribed with a different polymerase compared to mRNA, and they are not reverse transcribed by oligo-dT or gene specific primers [421]. Consequently, validation has to be performed to measure the variability of reference genes. If this is not done small but significant differences between study and control groups may be masked. Even worse, it is possible for artificial inter-group differences to be created if the reference gene is induced in one direction and the target gene is induced in the opposite direction (directional shift). It is extraordinary that despite these observations there are countless examples of published work that have used reference genes without any mention of a validation process.

An alternative is normalisation to total RNA. However, this method does not control for the RT and other steps, requires significant amounts of RNA, and there must be an accurate and reliable method of RNA quantification. This can be problematic when a spectrophotometer is used because of instrument insensitivity, signal contribution by contaminants (phenol in particular), residual DNA despite DNAase treatment and unreliability at concentrations below 100ng/uL [37, 39, 435]. In the current study, the Bioanalyser was found to give consistent results between group 1 and 2 genes and it is generally considered to be more reliable than a spectrophotometer [37, 39, 435]. However, the analyser is expensive and may not be widely available, especially to laboratories in developing countries where the burden of tuberculosis is the highest and there is an urgent need to build research capacity. The RiboGreen assay is widely regarded as the gold standard for RNA quantification. A non fluorescent dye binds to RNA and is quantified against a rRNA reference standard using a fluorimeter [451]. Results obtained using the RiboGreen assay and the Agilent Bioanalyser showed good correlation except at very low RNA concentrations (Prof. S.A. Bustin, personal communication).

For the proposed model of TB-specific cytokine study presented in this thesis, it was decided to choose the housekeeping gene method for normalisation as it is simple, least costly and measures the composite error of all the steps involved in the real-time PCR process. Finding a suitable HK (HuPO) created a convenient way to normalise for intra-experimental error and differing input amounts of RNA, yet concurrently avoids the variability associated with GAPDH and β -Actin. The disadvantage of HK gene validation is that considerable effort and cost is expended to perform an exercise like the one presented here. This may not be feasible in small studies or those with limited budgets. Similar constraints apply to normalisation with an average of 3 housekeeping genes [452], which is advocated by some investigators. Indeed, there are a number of programs (Gnorm, BestKeeper, Norm-Finder) based on the excel platform that allow the assessment of multiple reference genes by using the geometric mean of the candidate gene expression levels [452, 453]. Whilst this is a robust method for providing accurate normalisation it is severely limited by sample availability, labour intensity and cost. Consequently, the findings presented here will facilitate the use of the HK gene method in small laboratories or those from resource poor settings when studying TB host gene expression in blood and PBMC culture.

2.4.1.2. Choice of study participants and experimental design

In order to create maximum variability subjects of different ages, sex and ethnic backgrounds were used. Furthermore, different tissues or cell types sampled at different time points, which included cells challenged with TB antigen were used. The selection of subjects and time-points mirrored experimental protocols that will be used (described in chapter 3) to study IL-4. The HK gene variability in blood was largely due to inter-individual differences. There was surprisingly little difference in gene expression levels at four hours in the samples of both normal and tuberculosis patients, considering that these samples had been subjected to *in vitro* storage conditions for several hours. It is possible that the expression of highly regulated genes e.g. COX 2 would have shown a large variability between time points. Compared to blood, the variability in culture was due to both inter-individual differences and differences between different culture time points; these differences were not patient specific.

2.4.1.3. Screening of HK genes and selection of cut-offs

Using the ABI commercial plate was a convenient way to do the initial screen because these genes vary in expression levels and cover a wide range of biological functions. Group 2 genes were selected to increase the number of genes considered in the validation exercise.

A variability of two fold was chosen as some cytokines (e.g. IL-4) are both expressed and biologically functional at low levels; a one log difference being significant in human models of TB [17]. A HK gene with wider variability would increase the assay noise, hence limiting sensitivity. Consequently, defining the limits of the HuPO gene's variability is helpful for data interpretation of low copy number cytokines like IL-4 (for example a 5 fold difference between study groups may be statistically significant but not meaningful when the reference itself varies by 5 fold or more). It should be borne in mind that the resolution of the real-time PCR technique is dependent of the margin of error occurring during the multi-step process (variability

of the operator, the RT step and the reference). Consequently, when a change of ≤ 2 fold is detected, it may be statistically valid but is it meaningful? Without reference gene validation and other controls (standardisation of sample size or mass, using the same amount of input RNA in all reactions, robotic technology to minimise operator error) the resolution of the technique is unlikely to meaningfully detect small differences in gene expression (2 to 5 fold) [38, 435]. What constitutes an acceptable reference in relation to inter-group comparisons are discussed in the next section.

2.3.1.4. Conclusion: Reference gene validation

In conclusion HuPO is suitable for use as a house keeping gene in models of human pulmonary tuberculosis when gene expression is studied in whole blood and PBMC cultures. GAPDH and β -Actin are unsuitable for this purpose. Whatever strategy is used to control for differences in input RNA it must be validated for a particular experimental model on an individual basis. This is to avoid significant inaccuracies when quantifying target gene expression.

2.4.2. Effect of reference gene selection on inter-group comparisons

The former section outlined the selection of a suitable reference for normalising data pertaining to biological samples obtained from healthy volunteers and patients with TB. This section discusses how using this validated reference (HuPO), compared to an unvalidated one (GAPDH), impacts upon inter-group experimental comparisons and conclusions.

2.4.2.1. Change in target gene expression is reference dependent

The data already presented in section 2.4.1 demonstrate that many conventionally used reference genes vary widely in mRNA expression. What is discussed here is the effect of using a validated versus un-validated reference on the target gene expression. The error or 'noise' of an unvalidated reference may mask a significant inter-group difference or falsely create a difference where one is not present. The latter may occur when the target gene is induced in one direction and the reference is induced in the opposite direction. Indeed, figure 2.9 and 2.10 demonstrate that a significant increase

in IL-4 expression can be masked whilst a non-significant decrease in expression can be converted to a significant increase with an inappropriate reference gene. This may partly explain previous conflicting results when looking at IL-4 mRNA expression in TB [17, 27, 353]. Therefore, inappropriate normalisation can generate results that may be erroneous and not biologically meaningful.

Although it has been suggested that normalising to ribosomal RNA is a favourable strategy [37], the assumption that the rRNA:mRNA ratio is always constant is likely to be incorrect. This has been demonstrated in situations where there are proliferating cells and situations of stress (reviewed in [436]); it is therefore also possible in an infectious disease like tuberculosis. Consequently, normalization to rRNA will also require validation.

2.4.2.2. What constitutes an acceptable reference?

What is apparent from this study is that validated reference genes can be used to define the whole experimental error (error of all sequential steps of PCR including reverse transcription); however, this cannot distinguish between the experimental error and the inherent variability of the reference gene itself. Nevertheless, this does not matter as long as the cumulative error is less than the inter-group difference to be measured i.e. a reference gene that has a log variation is sufficient to measure a 2 log change in a gene of interest. Consequently, what constitutes acceptable variability of a reference is dependent on the inter-group difference being studied. Furthermore, it is crucial that both the directional shift and noise are defined for a particular reference gene prior to measuring the gene of interest.

2.4.2.3. Conclusion: Effect of reference gene selection on inter-group comparisons

In conclusion, this part of the study demonstrates that when a carefully validated reference is used to normalise data, biologically meaningful results can be measured. However, use of an unvalidated reference can lead to the generation of erroneous inter-group differences. Furthermore, in validating the reference gene the whole experimental noise can be assessed, thus defining the experiments sensitivity. Consequently, demonstrating the variation in expression of a reference gene and/or

the extent by which it may be affected by the experimental conditions is essential if robust and biologically meaningful conclusions are to be drawn.

2.5. Overall conclusions

The data presented in this chapter show that a 'one size fits all' approach (e.g. GAPDH and β -actin) to reference gene selection is inappropriate for data normalisation. If this approach is used then erroneous data are generated and the conclusions derived from inter-group study comparisons is misleading. Therefore, investigators must be able to demonstrate that the reference gene has been validated for the experiment in question. When whole blood and antigen driven PBMCs are used in samples acquired from index cases and healthy controls HuPO is a suitable reference as it has a average fold change of less than 2 fold.

The methods presented here have facilitated the development of a sensitive and specific assay to measure IL-4 and IL-4 δ 2 mRNA levels over a wide dynamic range (reproducible minimal detection limit of 10 copies and distinguishing an average 2 fold difference in target gene quantity between study groups). Furthermore, it ensures generation of data that can be interpreted with confidence and relevance. The specific reaction kinetics, detection limits, reproducibility, and primer and probe sequences of the real-time assay are outlined in chapter 3.

CHAPTER 3: GENERAL METHODS

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3.1. Patient selection and sample acquisition

3.1.1. Patients (details for HIV+ donors are outlined in chapter 8)

Twenty-nine patients with pulmonary tuberculosis (PTB), 15 of whom underwent lung bronchoalveolar lavage (BAL), were recruited at the Royal Free and Middlesex Hospitals, London, UK. All patients yielded positive cultures from sputum or alveolar lavage fluid, were negative for antibodies to HIV (3 ELISAs on 2 samples) and responded clinically to anti-TB treatment. All patients with PTB had pan-sensitive isolates, received standard short course chemotherapy (6 to 9 months) and responded clinically and radiologically to anti-TB treatment.

3.1.2. Control subjects, TB exposure and excluding LTBI

Control donors (n=21) were healthy volunteers matched to the first 21 sequentially recruited TB patients for age (within 4 years), sex and ethnicity. They were asymptomatic, had no risk factors for HIV infection (but were not formally tested), had normal chest x-rays and did not have LTBI despite risk of exposure to *M. tuberculosis* (TB health care workers and contacts of PTB patients with variable risk exposure; subjects with LTBI were excluded). Six of these volunteers underwent (BAL). Effector T-cell IFN- γ responses to TB specific antigens were used to confirm

absence of latent infection in the control subjects [41, 454]. Participants with a history of atopy or helminth infection, another inflammatory disorder, pregnancy or on immunosuppressive medication were excluded from the study. Informed consent was obtained from all patients and the Royal Free and UCLH ethical review committees approved the study.

3.1.3. Processing of whole blood samples

Whole blood (2.5 to 20 ml) was taken within the first 2 weeks of anti-TB treatment (baseline) and 2.5 ml was immediately transferred from the patient into PAXgene Blood RNA tubes (PreAnalytix, Qiagen, Cologne, Germany) to fix the mRNA profile [445]. The remaining blood was used for further experiments (cell sub-population studies, IFN- γ and IL-4 ELISPOT assays, mononuclear cell culture). Ten donors with PTB were bled after 3 months and then again within 4 weeks of stopping chemotherapy. Samples not immediately fixed in RNA buffer, were transported in a thermo flask at 37°C in accordance with regulations governing the transport of bio hazardous materials. A safety and hazards assessment was conducted for all experiments and the relevant good laboratory practice and category 3 laboratory training courses were undertaken. Methods relating to the processing of blood for half-life experiments and HIV+ subjects are described in chapter 4 and chapter 8, respectively.

3.1.4. Bronchoalveolar lavage

BAL was undertaken under local anaesthetic and midazolam sedation. To obtain BAL fluid 0.9% saline was instilled into a radiologically involved lung segment, using 180 ml saline in 60ml aliquots. In control donors the right middle lobe was lavaged. BAL fluid was transported on ice to a laboratory adjacent to the bronchoscopy suite, filtered through a 100 μ m Partec filter (CellTrics®, Münster, Germany) and centrifuged at 900 g for 3 minutes. A buffer containing chaotropic salts (RLT buffer, Qiagen) was added to the cell pellet to fix the RNA profile and the sample homogenised to disrupt cell structure (QIA shredder, Qiagen). The supernatant was frozen at -80°C and concentrated ~ 10 fold before analysis (Centriprep YM-3

Centrifugal Filter Unit, Millipore, Watford, England). An aliquot of fresh lavage fluid was used to determine BAL lymphocyte sub-populations by flow cytometry (section 3.5.).

3.2. Radiological scoring system for tuberculosis

To assess the extent of pulmonary disease a composite chest radiograph scoring system was developed, in collaboration with Dr J Haddock at the Royal Free Hospital, which divided the lung into 6 zones. The extent of airspace consolidation (0 to 3 based on the amount of shadowing in each zone), number of cavities (no cavities, <3 and > 3 in both lung fields) and extent of fibrosis (0 to 3 based on the amount of reticular shadowing) was assessed and scored (minimum score 0, maximum score 32) for each patient (appendix 1.1). Two radiologists blinded to the patient identities independently scored the X-rays; there was good inter-observer correlation ($p=0.0001$). To validate the x-ray scores post-treatment spirometry data (percent predicted forced vital capacity and forced expiratory volume in the 1st second; FVC and FEV1/FVC ratio) is being collected in an extended cohort of patients. These findings will be analysed when the cohort is large enough and the results will be published later. Although thoracic computer tomographic (CT) scans would be a better measure of disease extent and cavitation, they are not used in routine clinical practice, and their availability for this study was limited because of ethical and cost considerations. There are currently no validated scoring systems available for quantifying the radio-pathological changes seen in pulmonary tuberculosis.

3.3. Density centrifugation to obtain mononuclear cells

For cell separation and mononuclear cell culture experiments PBMCs were separated from fresh whole blood by density gradient centrifugation. Whole blood, after separation and removal of an aliquot of plasma, was layered (diluted 1:1 with Roswell Park Memorial Institute medium (RPMI)) over Ficoll-Paque Plus (sodium metrizoate/polysaccharide solution; Amersham Biosciences, Buckinghamshire, UK), and centrifuged at 900 g for 15 minutes (no brake); the PBMC layer was harvested with a Pasteur pipette. Red cells were lysed by incubation with ammonium chloride solution for 10 minutes on ice (StemCell Technologies, Vancouver, Canada). PBMCs

were washed twice in RPMI and then counted using a Neubauer counting chamber (depth 0.1 mm).

3.4. Cell separation to acquire whole blood cell sub-populations

Peripheral blood mononuclear cells (PBMC) from 10 patients with PTB and their healthy matched controls were used for cell sub-population studies. Enrichment of CD3+, CD3+CD4+ and CD3+CD8+ and depletion of T cells was performed using antibody based density centrifugation reagents, added to whole blood, according to the manufacturer's directions (RosetteSep, StemCell Technologies, Vancouver, Canada). The RosetteSep antibody cell separation cocktail consists of monoclonal antibodies to CD surface molecules and a tetrameric glycophorin antibody that agglutinates red blood cells and antibody bound leucocytes. The antibody bound cells are removed by density centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, UK). The negative selection T cell cocktail contains glycophorin A, anti-CD16, anti-CD19, anti-CD56 and anti-CD36 antibodies; the CD 4 cocktail in addition contains anti-CD8 antibody; the CD8 cocktail has in addition anti-CD4 antibody; total non T cells are acquired by depleting all CD3+ T cells. Cell viability was checked using trypan blue.

3.5. Flow cytometry

Cell sub-population purity and lymphocyte counts in BAL (total CD3+, CD4+ and CD8+) were confirmed by flow cytometry (10^4 gated events). Cells ($\sim 7.5 \times 10^5$) were stained with anti CD4-FITC, anti CD8-PE and anti CD3-PercP antibodies (BD Tritest™, BD Biosciences, Oxford, UK) and incubation on ice for 15 minutes. Excess antibody was removed by washing in FACS buffer (appendix 2) and cells were fixed in 1% formaldehyde. Mean cell purities of the relative whole blood fractions were > 95%, 99%, 93% and 90% for T, total non-T, CD4+ and CD8+ fractions, respectively and were determined by FACS using the Cell Quest 3.3 (FACScan; Becton Dickinson, San Jose, CA) and Ortho Cyturon absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ). Dot plots of the cell fractions are shown in chapter 6.

3.6. Immunoassays.

3.6.1. ELISPOT assay for IL-4 and IFN- γ

To determine whether healthy volunteers had latent TB infection, T cell IFN- γ responses to ESAT-6 and CFP-10 peptide pools were measured after ~16 hour incubation in serum-free medium using the T SPOT-TB kit (Oxford Immunotec, Oxford, UK) according to the manufacturer's instructions. PBMCs (2×10^6 cells/ml, 150 μ l final volume, 2×10^5 cells per well) were seeded into the wells containing pre-coated IFN- γ antibody. Responses, the number of IFN- γ spot forming T cells (SFC) per million PBMC was enumerated by a 'blinded' operator using an Oxford Immunotec ELISPOT reader. The assay contains an internal positive control (figure 3.1) defined, according to manufacturer's instructions, as < 20 spots in the phytohaemagglutinin (PHA) positive control wells (~ 100 spots/ 10^6 PBMC). Initially an in-house ELISPOT IFN- γ assay was developed using ESAT-6 protein, however, this assay was abandoned due to inconsistent results and poor reproducibility.

Duplicate IL-4 ELISPOT assays (significant if greater than 2 SD above the mean of unstimulated wells) were performed on ~ 2×10^5 PBMCs, non-T, T-cell, CD4+ T cell and CD8+ T cell fractions after ~ 48 hours incubation, in RPMI 1640 medium (supplemented with 5% human AB serum, glutamine and penicillin/streptomycin). Cells in positive control wells were stimulated with a combination of Phytohemagglutinin, Phorbol 12-Myristate 13-Acetate and calcium ionophore (final concentrations of 10, 0.5 and 0.5 μ g/ ml, respectively). Cell viability was assessed with trypan blue. IL-4 production was detected using 96 well ELISPOT plates (MultiScreen_{HTS} Filter Plates for Elispot, Millipore Corp.) coated overnight with 100 μ l anti human-IL-4 mAb (Mabtech, Stockholm, Sweden; 5 μ g/ml) and then blocked with RPMI containing 10% human AB. After 48 hours incubation the plate was incubated with 100 μ l biotin labelled anti-IL-4 antibody (1 μ g/ml, Mabtech) for 1 hour. Finally streptavidin alkaline phosphatase 1:10 000 (Caltag Laboratories, CA, USA), 100 μ l per well was added for 1 hour, followed by substrate solution (BCIP-NBT [5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium] conjugated to alkaline phosphatase, BioRad, CA, USA) for 15 minutes. The plate washed 5 times

between all steps using PBS (appendix 3). Spot counts expressed per million cells were analysed using the AID Elispot Reader System and software (AID, Strassberg, Germany).

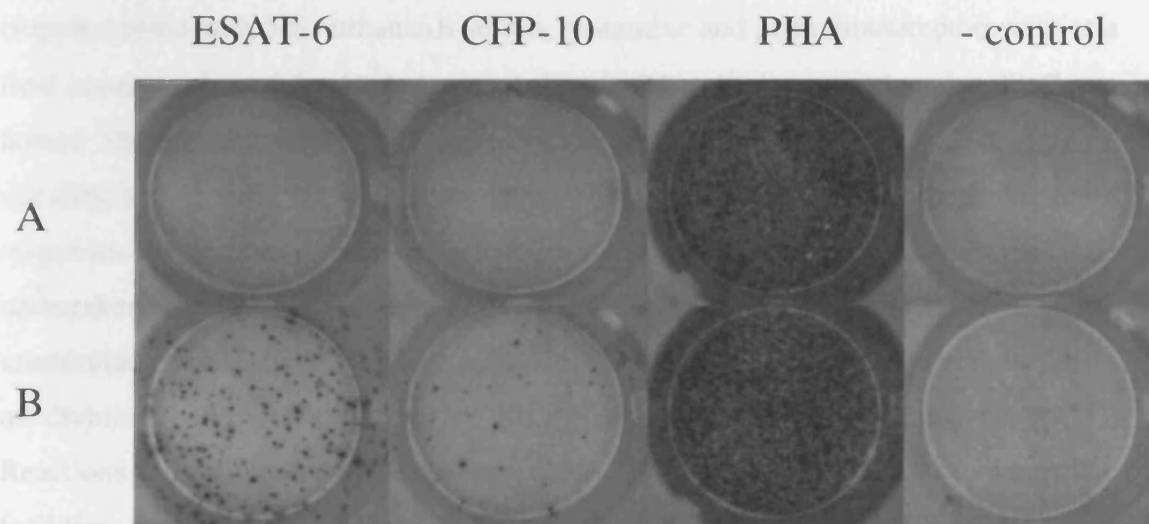


Figure 3.1. IFN- γ ELISPOT (T SPOT TB) reading of an individual with (row B) and without LTBI (row A). PBMCs are incubated with antigen (column 1 and 2 from the left), PHA (+ control in column 3) or no antigen (negative control, column 4). Detection of IL-4 by ELISPOT followed the same principle but wells were coated with anti-IL-4 antibody and the positive control was a combination of PHA, PMA and calcium ionophore.

3.6.2. Enzyme linked immunosorbent assays for IFN- γ , IL-4 and sCD30

Soluble CD30 (sCD30, Bender MedSystems, Vienna, Austria), IL-4 and IFN- γ (Pelikine CompactTM, Sanquin reagents, Amsterdam, The Netherlands) plasma and BAL levels were measured by ELISA in PTB and controls according to the respective manufacturers' instructions. The sensitivity of the assays were 0.33U/ml (sCD30), 0.5 pg/ml (IL-4) and 5 pg/ml (IFN- γ). Plates were read using an ELISA-plate reader

(MR5000, Dynatech, Guernsey). Standard curves were plotted and sample protein concentrations were calculated using Graphpad Prism.

3.7. Culture of peripheral blood mononuclear cells

Fresh PBMCs from 8 patients with PTB were cultured in filter sterilised RPMI (supplemented with 5% human AB serum, glutamine and penicillin/streptomycin) at a final concentration of 5×10^5 /ml, over 6 days in 24 well plates incubated at 37°C in a humid 5% CO₂ incubator; non adherent cells were harvested every 2 days. Cell viability was determined with 0.4% trypan blue (> 80% viability) and proliferative responses to antigen challenge (Cell Proliferation ELISA, BrDU, Roche, UK) undertaken on day 5 to 6 and expressed as a stimulation index relative to the unstimulated wells. BrDU is a thymidine analogue that is bound to the cellular DNA of dividing cells and detected by ELISA to a monoclonal antibody to BrDU. Reactions were stopped after 15 minutes using 1M sulphuric acid. Lack of appropriate facilities precluded the use of a radioactive 3-H thymidine proliferation assay. Experiments conducted with an aqueous calorimetric tetrazolium/formazan cell viability assay (CellTitre® 96 AQueous Non radioactive Cell Proliferation assay) was found it too be too insensitive for relatively low level of proliferation e.g. lymphocytes.

Cells were treated with crude whole cell sonicates of H37RV *M. tuberculosis* (50µg/ml final concentration), phytohaemagglutinin (5µg/ml), an environmental mycobacterium (sonicated *M. vaccae* NCTC 11659, 50µg/ml to determine if findings were TB specific) or medium alone. Antigens were prepared by sonicating (100-watt ultrasonic disintegrator with the wave peak distance set at 9 µm) harvested mycobacteria (grown on Sautons' slopes and centrifuged at 20,000 g for 15 min) for 15 min. The suspensions were centrifuged to remove cellular debris and the supernatants filter-sterilised (0.22 µm-pore size). The protein concentration was quantified by the Lowry method [455]. A single batch of sonicates was used for all reactions. Optimum antigen concentrations were dictated by performing preliminary experiments that included assessment of cell viability and proliferative responses. The

use of frozen PBMCs was abandoned after optimisation experiments revealed poor recovery of viable cells.

3.8. Real-time RT-PCR

RNA was isolated from whole blood using the Paxgene Blood RNA Kit (PreAnalytix, Qiagen) and from purified or cultured cells using the RNeasy® Mini Kit (Qiagen). All samples were treated with DNase (Qiagen). The RNA template was qualitatively assessed and quantified using an Agilent Technologies 2100 Bioanalyser (RNA 6000 Nano and Pico Labchip® [448]; the latter was used to quantify RNA amounts less than 5 ng; figure 3.2). Reverse transcription was performed using a fixed amount of input RNA (Omniscript™ for RNA derived from blood and mononuclear cell cultures and Sensiscript™, Qiagen for RNA derived from cell sub-population studies) and incubated at 37 °C for 1 hour, followed by 93°C for 5 minutes to denature the enzyme.

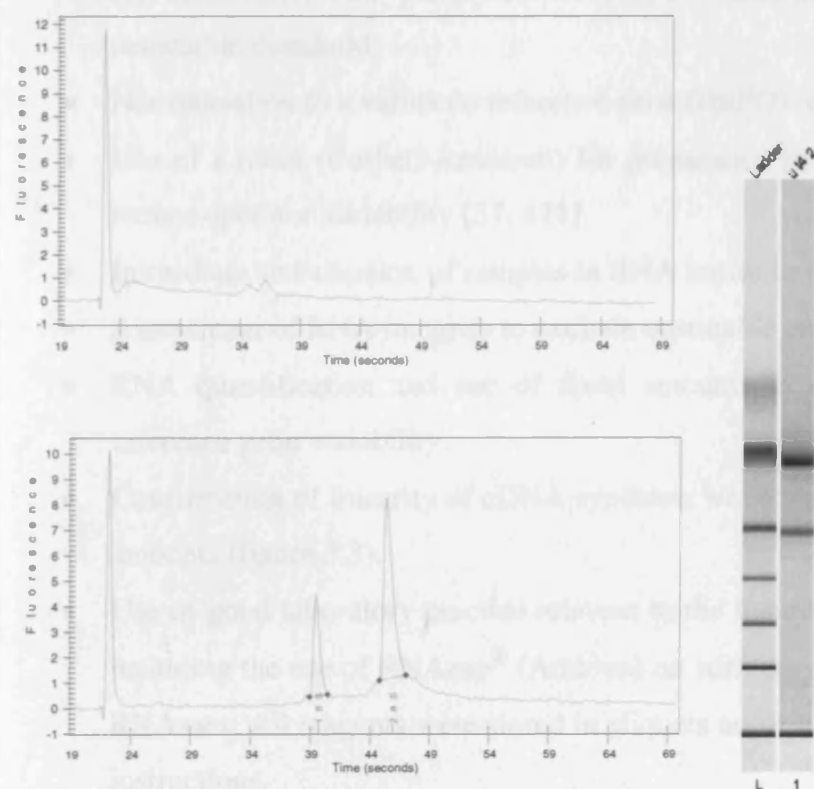


Figure 3.2. Assessment of RNA integrity using the Agilent 2100 Bioanalyser. The bottom tracing (fluorescence against time) shows good quality RNA whilst the top tracing shows poor quality RNA as reflected by the overall decrease in RNA signal, decreased ratio of 18s and 28s rRNA peaks and an abnormal baseline. An electronic capillary electrophoretic tracing shows the the 18s and 28s bands against a reference RNA standard (gel strip on the left).

Real time RT-PCR reactions were performed using a Rotogene real-time PCR system and Robot (Corrbet Research, Australia). Amplified templates were quantified using a standard curve prepared from 10 fold serial dilutions of purified PCR product (Qiagen). Measures taken to ensure robust and reproducible PCR data are shown in table 3.1 and figure 3.3. Reaction kinetics are outlined in table 3.2 and figure 3.4 and 3.5. Cytokine mRNA values were normalised to a validated housekeeping gene, human acidic ribosomal protein (HuPO) [409]. Raw data replicates that were non-reproducible and below the detection limit of the assay (10 copies) were given an arbitrary value of 1 copy in the final analysis. Primer and probe sequences are shown in table 3.3.

Table 3.1. Measures taken to ensure robust and reproducible real-time PCR data.

- A quantitative standard curve was run on every plate to monitor efficiency, reproducibility, inter-plate variability and exclusion of templates below a detectable threshold.
- Normalisation to a validated reference gene (HuPO) (chapter 2).
- Use of a robot (Corbett Research) for preparation of real-time PCR plates to reduce operator variability [37, 421].
- Immediate stabilisation of samples in RNA buffer to fix *in vivo* profiles.
- Assessment of RNA integrity to exclude unsuitable templates.
- RNA quantification and use of fixed amounts in each reaction to reduce reference gene variability.
- Confirmation of linearity of cDNA synthesis when using different initial RNA amounts (figure 3.3).
- Use of good laboratory practice relevant to the handling of RNA preparations including the use of RNAzap[®] (Ambion) on surfaces and plasticware to digest RNAases. All reagents were stored in aliquots according to the manufacturer's instructions.

Table 3.3. Reagent choice, primer concentration and performance influence of real-time PCR

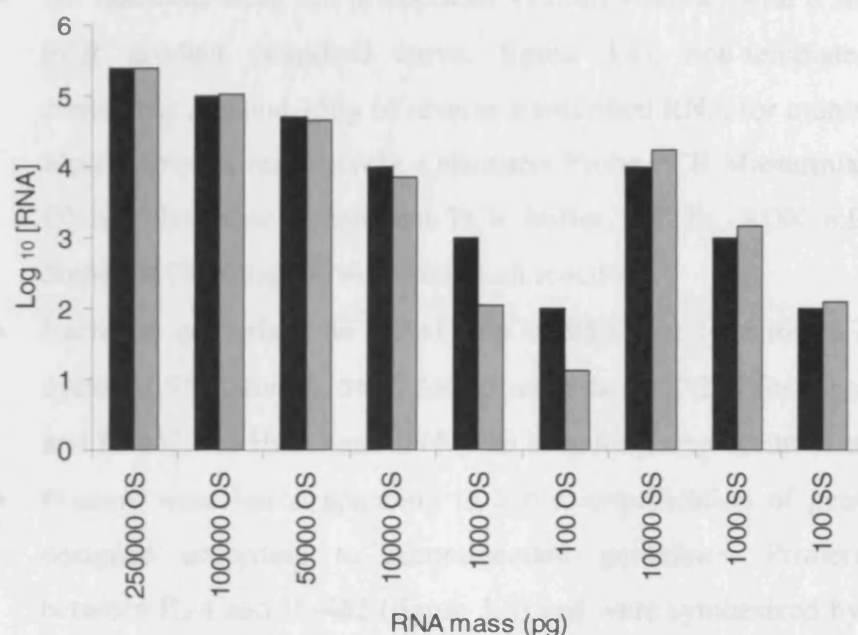
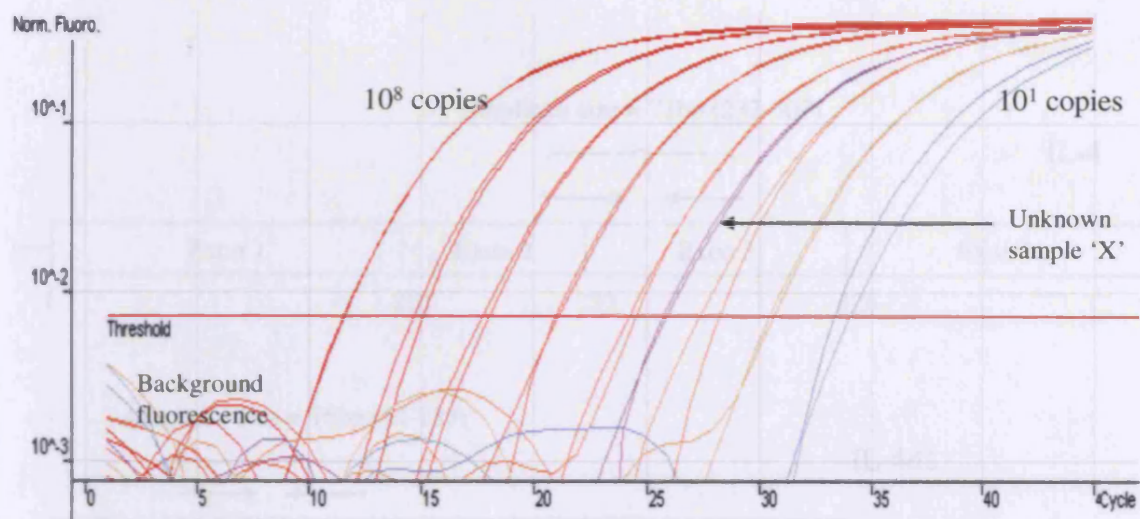


Figure 3.3. Linearity of the RT reaction for different input amounts of RNA. The copy number detected by real-time PCR (shaded bars) corresponded well with the RT input amount of RNA (copy number derived from the RNA mass; non-shaded bars) when using Omniscrypt™ (OS) down to 10000 pg but not below 1000 pg. This problem is resolved when Sensiscript™ (SS) is used instead; the 1000 and 100 pg [RNA] correlates well with the actual starting concentration.

Table 3.2. Reaction kinetics, primer concentrations and performance outcomes of real-time PCR.

- All reactions were run in duplicate (12.5µl volume) with 8 serial dilutions of PCR product (standard curve, figure 3.4), non-template controls, and containing 2ng and 15ng of reverse transcribed RNA for mononuclear cell and blood samples, respectively. Quantitect Probe PCR Mastermix (HotStarTaq™ DNA Polymerase, Quantitect PCR buffer, dNTPs, ROX reference dye and 5mM MgCl₂; Qiagen) was used in all reactions.
- Each run comprised an initial step of 95°C for 15 minutes followed by 40 cycles at 95°C for 5s, 56°C for 10 seconds and 72°C for 20 seconds for IL-4 and IL-4δ2 (for HuPO and IFN-γ the annealing step occurred at 55°C).
- Primers were intron spanning to avoid amplification of genomic DNA and designed according to recommended guidelines. Primers distinguished between IL-4 and IL-4δ2 (figure 3.5) and were synthesized by MWG Biotech (Germany). The coding regions chosen for amplification are not known to be associated with IL-4 gene polymorphisms [456].
- Primer and probe concentrations were optimised to achieve efficiencies approaching 100% for all amplicons (final primer concentrations were 500 nM for IL-4 and IL-4δ2 and 250 nM for IFN-γ and HuPO; all probes had a final concentration of 500 nM). All amplicons were run on an agarose gel (appendix 4) to exclude primer dimers. Reaction specificity (amplification of the correct product) was confirmed using Agilent 2100 Bioanalyser; in addition PCR products for IL-4, IL-4δ2 and HuPO were sequenced.
- Reproducibility (inter-experiment variability) was < 20% (co-efficient of variation) for IL-4, IL-4δ2, IFN-γ and HuPO (CV calculated using raw copy numbers of 5 reactions done over a 6 month period and using the same template; data for IL-4 is shown in figure 3.6).
- The minimum reproducible detectable threshold was 10 copies for IL-4, IL-4δ2 and IFN-γ. Copy numbers were regarded as valid if replicates were within 30% of each other.



Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
IL-4	Standard	11.64	100,000,000.00	104,946,255.19	4.95
IL-4	Standard	11.68	100,000,000.00	102,123,758.13	2.12
IL-4	Standard	15.15	10,000,000.00	9,265,994.04	7.34
IL-4	Standard	14.74	10,000,000.00	11,091,739.15	10.92
IL-4	Standard	17.82	1,000,000.00	1,089,529.70	8.95
IL-4	Standard	18.14	1,000,000.00	948,087.16	5.19
IL-4	Standard	21.22	100,000.00	101,282.04	1.28
IL-4	Standard	21.11	100,000.00	109,700.15	9.70
IL-4	Standard	24.32	10,000.00	10,673.25	6.73
IL-4	Standard	24.62	10,000.00	8,573.39	14.27
IL-4	Standard	28.29	1,000.00	900.23	9.98
IL-4	Standard	27.47	1,000.00	1,092.38	9.24
IL-4	Standard	30.77	100	99.76	0.24
IL-4	Standard	30.59	100	113.45	13.45
IL-4	Standard	34.18	10	8.38	16.2
IL-4	Standard	33.45	10	11.32	13.2
Sample 'X'	Unknown	26.04		3,078.23	
	Unknown	25.84		3,558.83	
	NTC				
	NTC				

Figure 3.4. IL-4 dilution series and quantification of unknowns. A 10 fold serial dilution of standards were co-amplified with unknown samples (sample 'x' in this example) on every plate. The minimal reproducible detectable number was 10 copies for both IL-4 and IL-4δ2; detection of 1 copy of IL-4 was stochastic. The adjoining table (in Rotorgene result format) shows the calculated copy numbers (Calc Conc) derived from the standard curve (99.1% efficiency); the calculated copy numbers for sample 'x' are shown in blue and negative controls in red text.

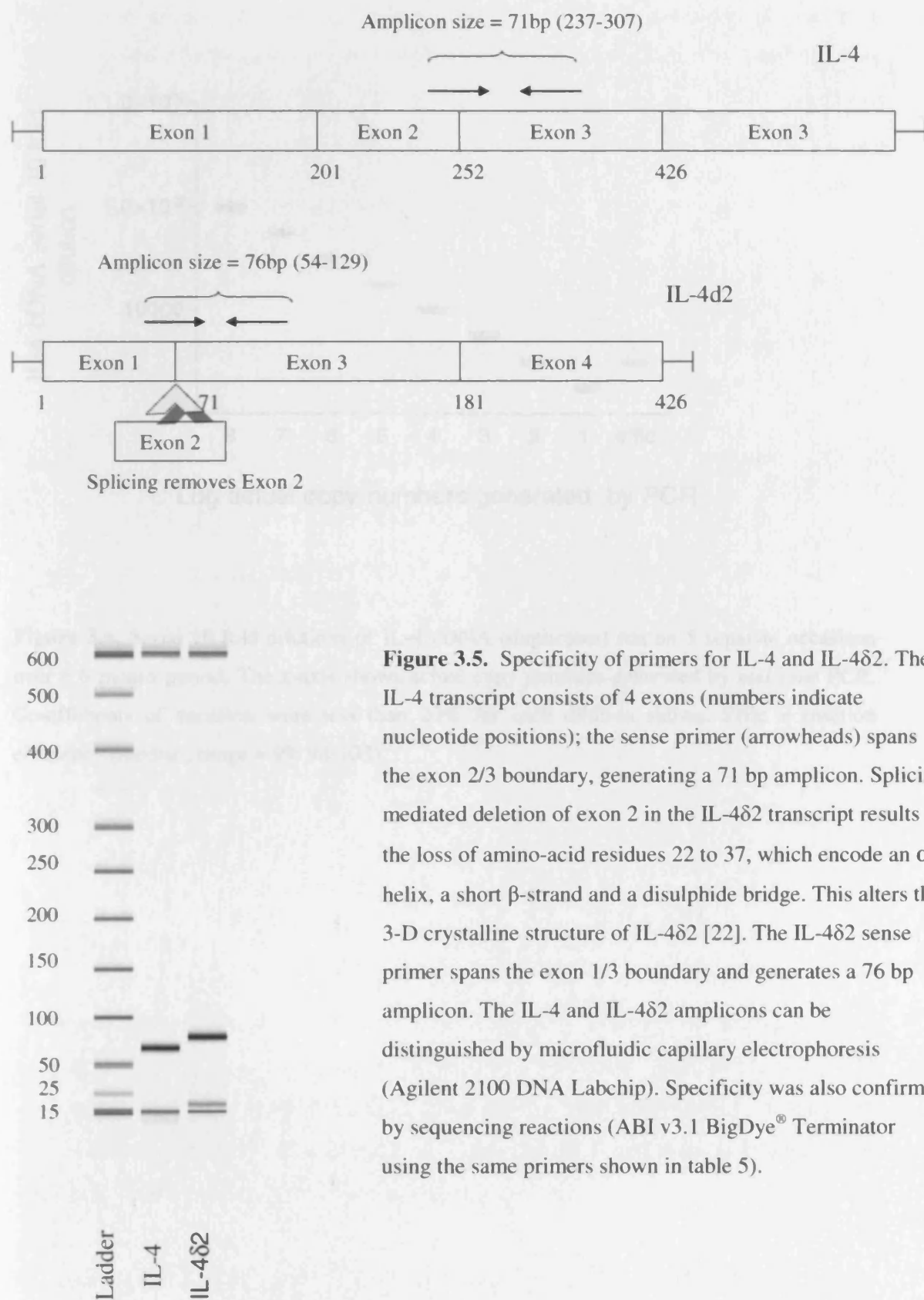


Figure 3.5. Specificity of primers for IL-4 and IL-4δ2. The IL-4 transcript consists of 4 exons (numbers indicate nucleotide positions); the sense primer (arrowheads) spans the exon 2/3 boundary, generating a 71 bp amplicon. Splicing mediated deletion of exon 2 in the IL-4δ2 transcript results in the loss of amino-acid residues 22 to 37, which encode an α -helix, a short β -strand and a disulphide bridge. This alters the 3-D crystalline structure of IL-4δ2 [22]. The IL-4δ2 sense primer spans the exon 1/3 boundary and generates a 76 bp amplicon. The IL-4 and IL-4δ2 amplicons can be distinguished by microfluidic capillary electrophoresis (Agilent 2100 DNA Labchip). Specificity was also confirmed by sequencing reactions (ABI v3.1 BigDye[®] Terminator using the same primers shown in table 5).

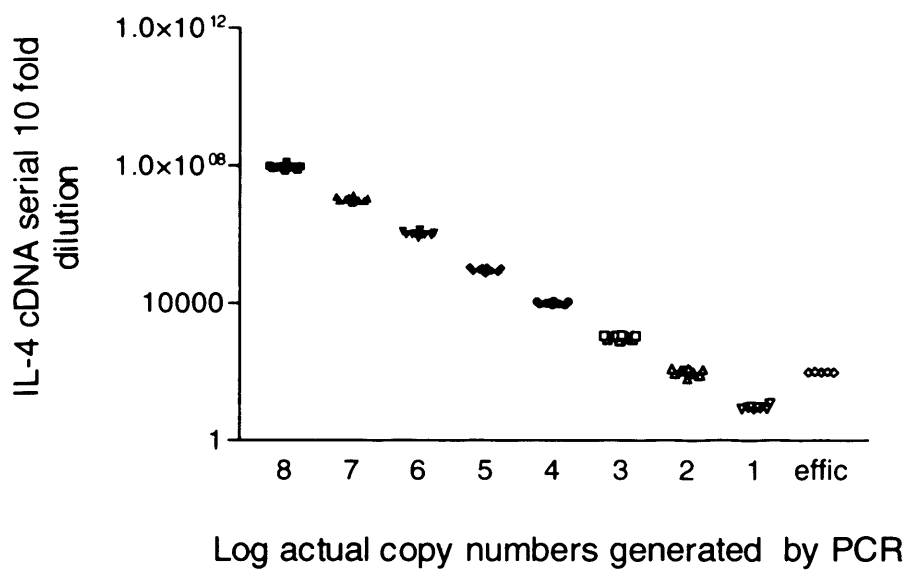


Figure 3.6. Serial 10 fold dilutions of IL-4 cDNA (duplicates) run on 5 separate occasions over a 6 month period. The x-axis shows actual copy numbers generated by real-time PCR. Co-efficients of variation were less than 20% for each dilution shown. Effic = reaction efficiency (median; range = 99; 98-103).

Table 3.3. Primer and probe sequences used to quantify gene expression by real-time PCR. Primers and probes for each gene were designed with the assistance of primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Amplicon size was kept to ~ 100 bp for maximal amplification efficiency.

Genes	* Probe sequence 5'-(FAM- TAMRA)-3' ◇ primer 1 -5'-3' □ primer 2 -5'-3'	Product size	R ²
IL-4	* AAACCTTCTGCAGGGCTGCGAC ◇ GCTGCCTCCAAGAACACAAC □ CTGTAGAACTGCCGGAGCAC	71bp	0.998
IL-482	* AAACCTTCTGCAGGGCTGCGAC ◇ GCCTCACAGAGCAGAAGAACAC □ CTGTAGAACTGCCGGAGCAC	76bp	0.999
HuPO	* TGCCAGTGTCTGTCTGCAGATTGG ◇ GCTTCCTGGAGGGTGTCC □ GGA CTCGTTTGTACCCGTTG	105bp	0.999
IFN- γ	* TGGCTGTTACTGCCAGGACCCA ◇ TTCAGCTCTGCATCGTTTTG □ TCCGCTACATCTGAATGACCT	112bp	0.998

3.9. Data analysis

The study was designed (prospective cross sectional) to detect an inter-group (12 patients per group) IL-4 copy number difference of ~1.5 logs (standard deviation 1.5) with a power of 80% at the 5% significance level. Statistical methods and sample sizes were determined Dr Morris and Dr F Lampe of the Department of Primary Care and Population Sciences, Royal Free Hospital, London.

TB patient and control groups were compared using the Mann-Whitney U test. Wilcoxon matched pairs test was used to assess changes within groups over time. Spearman rank-sum correlation was used to assess associations between variables. To

compare TB patients and controls with respect to T-cell IL-4 production, while controlling for non-T cell IL-4 production, linear regression was used on logged data. Cytokine ratios were expressed as log differences between respective cytokine pairs. Analysis was conducted using GraphPad prism and SPSS v 11.5.

CHAPTER 4: HALF LIFE OF IL-4 AND IL-4δ2

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4.1. Introduction

Th2 cytokines enhance disease susceptibility and progression by subverting macrophage protective mechanisms, and exacerbate immunopathology by enhancing the toxicity of TNF (reviewed in detail in chapter 1 and in [31]). Whilst real-time PCR allows reliable quantification of steady state IL-4 mRNA levels it tells the investigator nothing about transcription rates or mRNA degradation (mRNA stability or half-life). Regulatory mechanisms like soluble IL-4 receptor [308] and natural antibodies [311, 313] may modulate the biological effects of IL-4 (discussed in chapter 1, section 1.6.4.1). An additional putative mechanism of regulation, about which little is known, is enhancement or inhibition of IL-4 mRNA stability. Logically, enhancement of stability would be expected to facilitate the immunopathologic effects of IL-4. Indeed, even if mRNA half life is modestly prolonged (~ four fold) this can result in large increases in mRNA (~1.5 logs) and sustained cellular effects [457]. However studies of IL-4 mRNA stability in human biological samples are sparse [297, 412, 458, 459] and there are no data on whether mRNA stability is modulated in subjects with pulmonary tuberculosis compared to healthy volunteers.

A number of factors (sequences within the mRNA molecule, proteins, enzymes etc) either alone or in combination may modulate the stability of human mRNA (these factors are summarised in table 4.1).

Table 4.1. Factors and motifs that determine mRNA stability. One or more motifs may interact in a compartment and cell specific manner to regulate mRNA stability.

- Sequences in different parts of the mRNA molecule (sequence determinants)
 - poly (A)
 - 3' untranslated region (heavily influences half-life [457])
 - * AU-rich elements (AURE; translocation of AURE from one mRNA to another dramatically increases instability [457])
 - * Iron responsive elements (sequences in the 3' end determining transferrin mRNA stability)
 - * stem loops (IGF I and histone mRNA)
 - 5' untranslated region and mRNA cap
 - coding region
- mRNAases (poorly studied and incompletely characterized)
- RNA binding proteins
 - to AU rich regions
 - poly (A) binding proteins
 - Others (Iron regulatory protein, c-fos and c-myc coding region determinant-binding proteins, ribonucleotide reductase m-RNA binding proteins)
- Non-RNA binding proteins
 - β tubulin, histones, HSP, p27^{rex}, factors affected by translational inhibitors etc
- Hormones, GF, ions
 - Estrogen, phorbol esters, cytokines, growth factors, kinases
- Association with ribosomes and translation inhibitors

One important determinant of stability is the presence of AU (adenylate-uridylate) rich elements in the 3' untranslated region of the mRNA molecule. Recently a new class of therapeutic agents, p38 mitogen activated-kinase (MAPK) inhibitors belonging to the pyridinyl imidazole class of compounds, were discovered. They have anti-inflammatory effects in murine models of rheumatoid arthritis [460] and ischemia reperfusion injury [461], and exert their effects by phosphorylating mRNA AU binding proteins, thereby reducing the mRNA stability of cytokines like IL-3, IL-6, IL-8 and TNF (reviewed in [298]). This may conceivably have beneficial outcomes on the immunopathology of tuberculosis by effects on multiple cytokines including IL-4. However, there are no studies examining whether the stability of human IL-4 induced by TB antigen is MAP kinase dependent.

To address these questions the half-life of IL-4 mRNA, and of the antagonist, IL-4 δ 2, was evaluated in the blood of patients with active TB compared to healthy volunteers, and in TB antigen driven THP1 cells treated with an inhibitor of p38 MAP kinase.

4.2. Methods

4.2.1. Patients and samples

Eight patients with pulmonary tuberculosis (sputum culture positive, negative for antibodies to HIV, mean age 33 years, 5 males, mixed ethnicity- 4 Black Africans, 2 Somalians and 2 whites) and 5 healthy volunteers (mean age 34 years, 3 males, mixed ethnicity- 1 Indian, 1 Korean, 1 Chinese, 2 White) were recruited at the Royal Free and Middlesex Hospitals, London, UK. Control subjects had previous exposure to *M. tuberculosis* but no LTBI, which was excluded by determining TB antigen-specific (ESAT-6 and CFP-10 peptide pools) IFN- γ ELISPOT responses in PBMCs as outlined in chapter 3.

Samples (20 ml whole blood) was acquired before or within the 1st 2 weeks of anti-TB treatment. After phlebotomy 2.5ml was immediately transferred into a PAXgene Blood RNA tube (PreAnalytix, Qiagen, Cologne, Germany) to fix the baseline mRNA profile [445]. Actinomycin-D was added to a 12ml aliquot of

heparanised blood and transported, in a thermoflask at 37°C, to the laboratory where 2.5ml aliquots were transferred to a PAX tube at different time points. The blood was stored in a humid 5% CO₂ incubator during all experiments.

4.2.2. Inhibition of mRNA transcription

All blood samples were treated with 5 µg/ml Actinomycin D (ActD, Sigma-Aldrich, Dorset) and samples harvested at 0, 60, 120 and 240 minutes. These time-points proved unsatisfactory for the measurement of the half-life of IL-4δ2 mRNA, which in control subjects was found to be less than 1 hour. Consequently, in 5 control subjects blood samples were also harvested at 15, 30, 45, 60 and 120 minutes. Optimisation experiments conducted using different ActD concentrations (5, 10 and 20 µg/ml) revealed no significant differential effect on IL-4 and IL-4δ2 mRNA half-life. To verify that ActD effectively inhibits cellular transcription preliminary experiments were performed using naturally unstable mRNA (c-myc) as a positive control.

4.2.3. THP1 cell line and p38 MAP kinase inhibitor

The human myelomonocytic cell line THP1 (American Type Culture Collection, Manassas, Va.) was cultured (2×10^6 /ml final concentration) in filter sterilised medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin [Gibco BRL, Paisley, UK] and 10 % fetal bovine serum) at 37°C using a 5 % CO₂ incubator. For the induction of macrophage cell differentiation 2×10^5 /ml cells were initially propagated in 250 ml flasks (Nunc) for 2 days and then treated with 1.2% of dimethyl sulphoxide (DMSO, Sigma-Aldrich, Dorset) for 24 hours and then washed twice in medium. After viability assessment using trypan blue cells were incubated with 90 µg/ml sonicated *M. tuberculosis* for 6 hours (designated baseline). The concentration of TB antigen was determined in optimisation experiments that allowed induction of immunological markers without cell toxicity. For the measurement of total RNA half life ($T_{1/2}$), stimulated cells were treated at baseline with 10 µg/ml ActD and with or without SB202190 (Calbiochem; a potent and cell-permeable inhibitor of p38 MAP kinase). Cells were harvested at baseline, 30, 60, 120, 240 and 480 minutes. Osteogenic differentiation factor (SOX 9) gene

expression was used as a positive control as its half-life is known to be p38 regulated in THP1 cells [297].

4.2.4. RNA extraction, RT and real-time RT-PCR

RNA was isolated from whole blood using the Paxgene Blood RNA Kit (PreAnalytix, Qiagen) and from cultured cells using the RNeasy® Mini Kit (Qiagen). Quality assessment of RNA, DNAase treatment, RT and real-time PCR was performed as outlined in chapter 3. The 5' 3' forward and reverse primer and probe sequences for SOX-9 were AAGCTCTGGAGACTTCTGAACG, GTAATCCGGGTGGTCCTTCT and CTCCTCCACGAAGGGCCGCT, respectively.

4.2.5. Data analysis

Depending on the shape of the decay curve conventional linear regression may over or under-estimate the half-life. Consequently, one phase exponential phase decay nonlinear regression (healthy controls and THP1 cells) or half life cut points from the actual curves (TB patients) was employed to measure the half life of IL-4 and IL-4δ2. TB patient and control groups were compared using the statistical methods outlined in chapter 3.

4.3. Results

4.3.1. IL-4 mRNA half-life in whole blood

The median (range minutes) half-life of IL-4 mRNA was significantly prolonged in patients with tuberculosis compared with control subjects [210 (145- 288) vs 49 (39-66), ~ 5 fold increase, $p= 0.0016$; figure 4.1 A and 4.2]. By contrast, in donors with tuberculosis compared to healthy volunteers the half-life of IL-4 δ 2 mRNA was not prolonged [(43 (30-72) vs 33 (20-62); $p= 0.7$; IL-4 δ 2 was detected in only 4 patients and 5 controls; figure 4.1 B and 4.2]. Consequently, the median (range) IL-4/ IL-4 δ 2 half-life ratio in 8 TB patients was significantly greater than in the 5 control subjects [1.6 (0.5-2.4) vs 0.09 (-0.04 -0.5), $p= 0.003$, figure 4.3]. There was no correlation between IL-4 mRNA half-life and disease extent on chest x-ray or presence of radiological cavitation.

4.3.2. Th2/Th1 ratio in whole blood

In *unstimulated* cells in whole blood the baseline Th2/Th1 (IL-4/IFN- γ) ratio was significantly higher in TB patients compared to controls [2.5 (-0.4- 2.8) vs 0.3 (-0.3-1.1), $p= 0.03$, figure 4.4].

4.3.3. p38 MAPK inhibitor and IL-4 mRNA levels in THP1 cells

The mRNA stability of SOX-9 (mean of duplicate wells), but not that of IL-4, was p38 MAPK dependent in THP1 cells incubated with ActD and TB antigen *in vitro* (median mRNA half-life in p38 treated vs untreated wells was 36 vs 28 minutes for IL-4 and 10 vs 134 minutes for SOX 9, respectively).

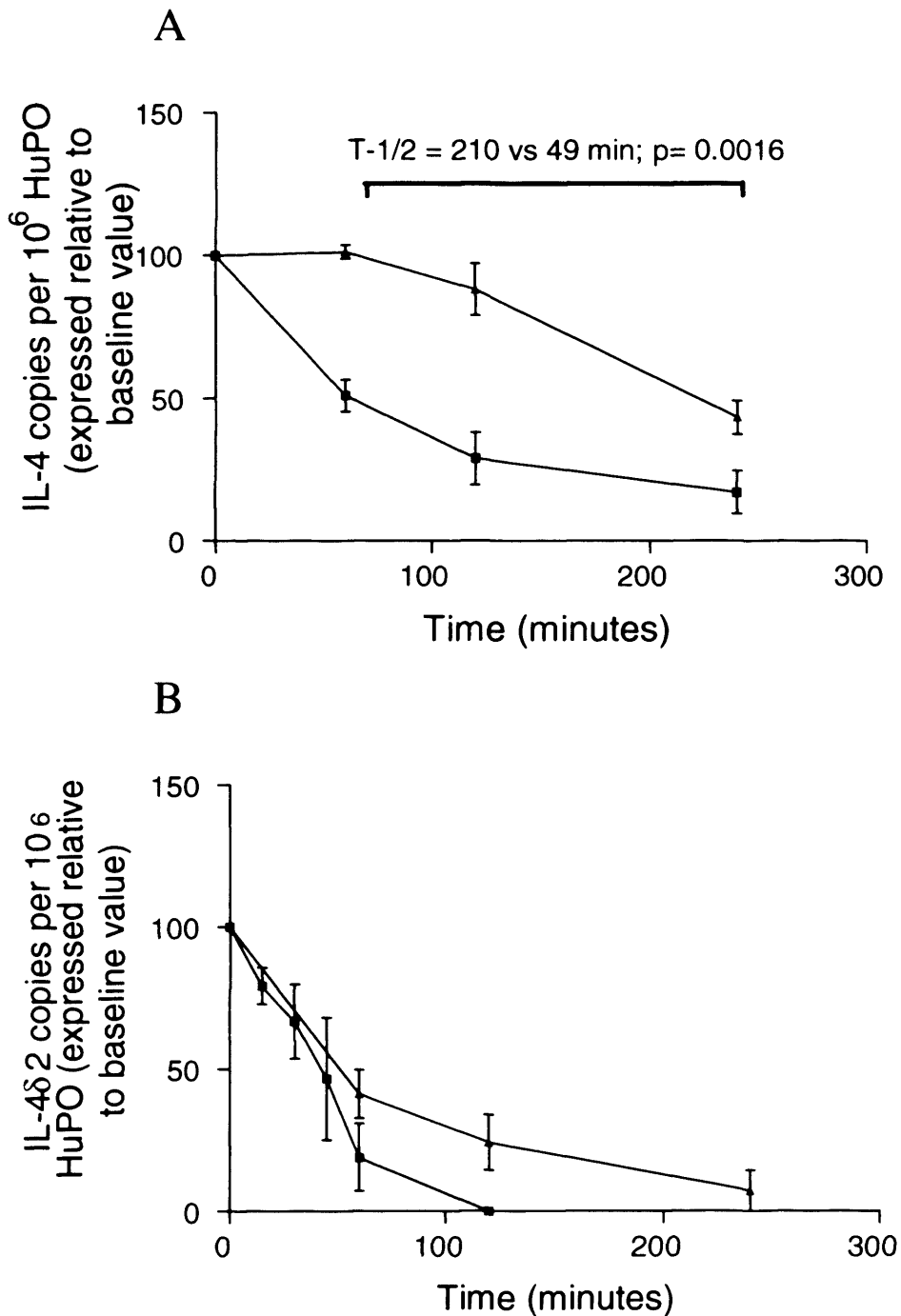


Figure 4.1. mRNA stability over 240 minutes of IL-4 (1A) and IL-4 δ 2 (1B) in donors with pulmonary tuberculosis (n=8) and healthy volunteers (n=5). IL-4 mRNA decay was significantly slower in patients (T_{1/2}= 210 minutes, triangles) compared to controls (T_{1/2}= 49 minutes, squares), p= 0.0016 (1A). By contrast, the IL-4 δ 2 mRNA stability was similar in both groups (1B). Error bars represent SEM.

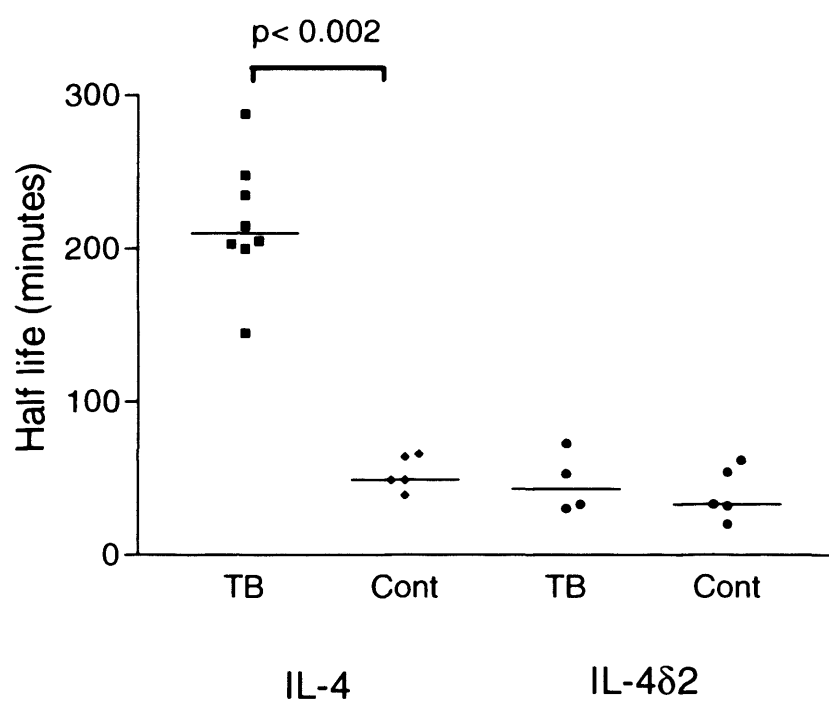


Figure 4.2. Scatter plot showing the whole blood mRNA half life of individual patients with pulmonary tuberculosis and healthy volunteers. The half-lives show relatively little spread in patients and controls. Horizontal lines represent medians.

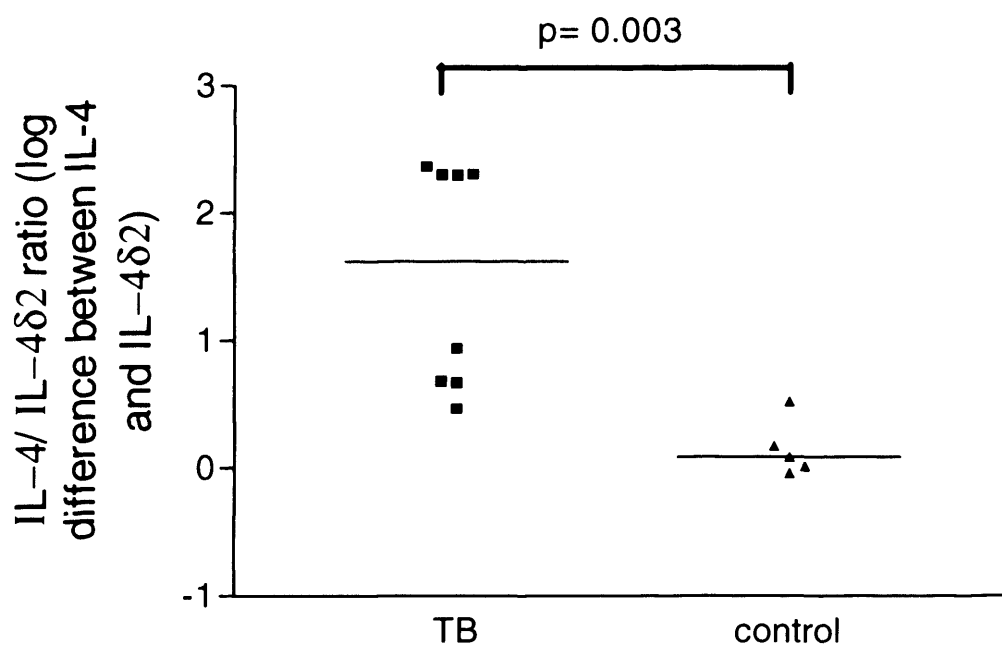


Figure 4.3. Whole blood IL-4/IL-4 δ 2 ratios in donors with tuberculosis and healthy controls.

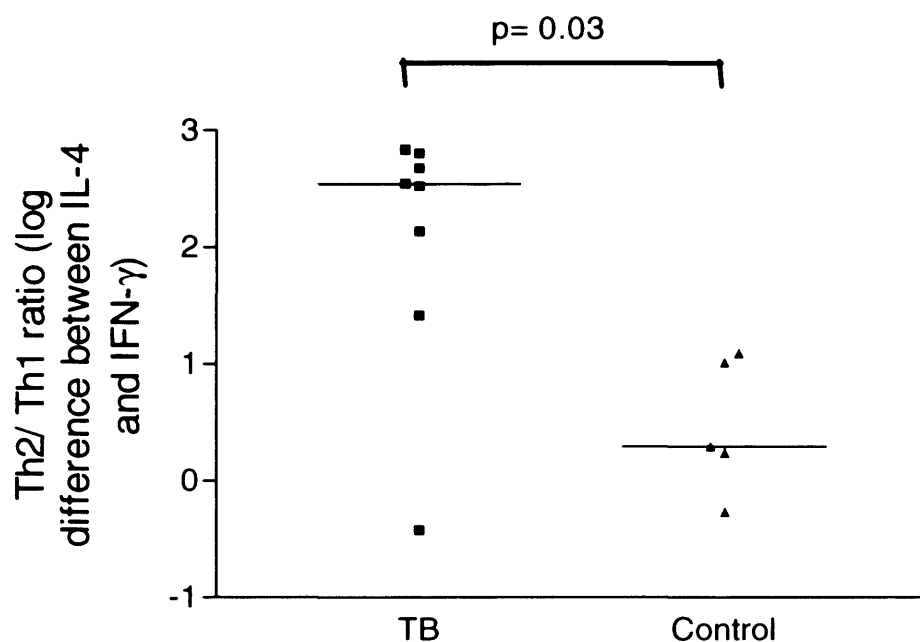


Figure 4.4. Th2/Th1 (IL-4/IFN- γ) ratio of unstimulated cells in whole blood from donors with pulmonary tuberculosis and healthy volunteers.

4.4. Discussion

A study of human mRNA stability showed significant half-life prolongation (~ 5 fold) of the prototype Th2 agonist, IL-4, but not of the antagonist, IL-4 δ 2, in donors with pulmonary tuberculosis compared to healthy controls. Consequently, the IL-4/IL-4 δ 2 half-life ratio was significantly higher in TB compared to healthy volunteers. This is the first study to show enhanced stability of a gene of interest compared to its splice variant. Furthermore, enhanced stability of IL-4 in whole blood, ex vivo, has not previously been described in a specific human disease.

4.4.1. IL-4 and IL-4 δ 2 mRNA half life

With respect to human TB these data suggest a hitherto undescribed post-transcriptional regulatory mechanism that may potentiate the biological effect of IL-4. Alternative splicing of mRNA encoded by a single gene is a recognised regulatory mechanism that controls gene expression and functional diversity of proteins [319,

325]. However, the increased IL-4/ IL-4 δ 2 half-life ratio in TB illustrates that differential stability of mRNA variants of a single gene may further modulate gene expression. IL-4 may subvert protective mycobactericidal mechanisms by down regulating protective innate responses (reviewed in chapter 1 and [236]). The association between IL-4 and immunopathology has been shown in murine models [14, 16, 364] and human studies (discussed further in relation to data presented in chapter 5). These deleterious effects are likely enhanced because the half-life of the antagonist, IL-4 δ 2, remains unchanged.

The interaction between IL-4 and IL-4 δ 2 is likely to have significant applicability to tuberculosis as contacts of index cases who remain healthy have high levels of the antagonist, IL-4 δ 2, but not of the agonist, IL-4 [360, 361]. Furthermore, it is demonstrated that unlike IL-4, IL-4 δ 2 mRNA levels increase with clinical improvement (chapter 5) and TB antigen preferentially drives IL-4 but not IL-4 δ 2 mRNA expression in mononuclear cell cultures from TB patients (chapter 7).

It remains unclear whether the enhanced IL-4 mRNA stability is TB-specific compared to other diseases where Th2 cytokines are prominent and associated with immunopathology or fibrosis. There are no data on cytokine mRNA stability in Th2-biased conditions such as idiopathic pulmonary fibrosis or leprosy. However, in a murine model of hypersensitivity pneumonitis, Th2 rather than Th1 biased mice had enhanced IL-5 mRNA stability [42]. The data presented here do not show a direct association between enhanced IL-4 mRNA stability and immunopathology (radiological disease scores) but it is possible that such a relationship would have been demonstrated in a larger cohort of patients. Nevertheless, similar to the murine pneumonitis model [42], patients with TB who had a Th2 biased profile, unlike healthy volunteers who did not, showed enhanced IL-4 mRNA stability.

4.4.2. p38 MAPK inhibitors and mRNA half-life

The mechanism by which this differential alteration in mRNA stability occurs in TB remains unclear. Certain mRNAs have AU rich motifs (contain AU proteins) in the 3' region. In some cases (e.g. IL-6 mRNA) p38 MAP kinases phosphorylate AU

binding-proteins, thereby impeding translation and reducing mRNA stability [298, 457]. Approximately 900 AU-motif-containing genes have been described but only a minority (~50) are p38 MAPK regulated [298, 457]. The anti-inflammatory potential of p38 MAPK inhibitors, the only known therapeutic modulator of mRNA stability, has recently been explored in human phase 1 studies [462] and disease models of rheumatoid arthritis [460]. It is intriguing to speculate that reducing the stability of IL-4, which is a key facilitator of immunopathology in tuberculosis (reviewed in [12, 236]), may impact on morbidity and disease progression, as it does in models of unrelated inflammatory diseases [460, 461].

However our data show that, unlike SOX-9, IL-4 mRNA stability does not appear to be p38 MAP kinase dependent in TB antigen stimulated THP1 cells, or for that matter in LPS stimulated THP1 cells [297]. Discordant data have been reported when TNF mRNA stability was studied in models using inhibitors of p38 MAPK [297]. Collectively, these data suggest that stability may depend on a number of factors including cell type (human versus murine), inducing antigen (LPS versus other) and interaction with other mRNA stability determinants (described in table 4.1 and reviewed in [457]). Consequently, it remains unclear whether IL-4 mRNA stability is p38 MAPK independent in specific cells types, which show increased IL-4 mRNA expression in TB (chapter 6). p38 MAPK has been shown to interact with NF-kappa B and modulate the levels of a number of inflammatory molecules [297, 298] and it is therefore still possible that it may have a modulatory effect in TB.

4.4.3. Validity of results

Are the observed 2 to 3 fold changes over the course of individual mRNA half-life decay curves and the five fold inter-group changes in mRNA stability, technically valid when using real-time PCR? Although real-time PCR is the most sensitive and reliable means to quantify gene expression the measurement resolution of this technique remains contentious. To keep measurement error to a minimum we used a reference validated for our experimental conditions (including use of blood from index cases and volunteers and TB antigen stimulated cells) [409], quality assessed all RNA templates, used a set amount of RNA in all reactions, immediately fixed mRNA profiles *ex vivo*, and employed a robot to minimise operator error. Furthermore, the

pattern of mRNA decay curves were consistent in each group of participants (TB or controls). Consequently, we feel that our results are reliable. Indeed, when measurement error is carefully controlled for, reliable detection of as little as 2 fold have been reported with real-time PCR [421]. Our data also indicate that the reference gene (HuPO) has a relatively long half-life and remained stable over the first 240 minutes. Analysis of transcripts with a longer half-lives requiring the use of later time points would necessitate an alternative normalisation strategy (e.g. total RNA [37]).

A limitation of the study is that control subjects were incompletely matched to patients with respect to ethnicity. However, the variable ethnicity of the study subjects makes these results more generalisable to an ethnically diverse population that is encountered in many large cities in both developed and developing countries. Nevertheless, it is possible that the results may have been different in a single ethnic group or in a developing country setting.

4.5. Conclusion

The half life of IL-4 mRNA, but not that of the natural antagonist IL-4 δ 2, is prolonged in pulmonary tuberculosis. This may represent an additional post-transcriptional regulatory mechanism by which *M. tuberculosis* may subvert host immune responses. Further studies are required in TB and other infectious diseases to determine whether pathways that modulate mRNA stability may offer future therapeutic potential.

CHAPTER 5: IL4, IL4 δ 2, IFN- γ AND SCD30 EXPRESSION IN WHOLE BLOOD & LUNG LAVAGE AND CORRELATION WITH CLINICAL PARAMETERS

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5.1. Introduction

Immunity to human mycobacterial disease is known to require TNF- α and a Th1 response because genetic defects in signalling by IFN- γ or IL-12 lead to susceptibility [343, 344] and neutralisation of TNF- α reactivates latent infection [153]. Although these data suggest a minimum requirement for Th1 cytokines and TNF- α , it is becoming evident that is not the complete answer. There are strong IFN- γ responses at the site of disease and observations about the protective efficacy of vaccines, and pathological changes in TB lesions, suggest that augmenting Th1 responses are unlikely to improve protective efficacy but will nonetheless enhance immunopathology (discussed later in this chapter). Indeed immunopathology is essential to the pathogenesis of *M. tuberculosis* because cavities opening into bronchi allow the dissemination of bacilli by coughing.

These and other considerations have led several authors to postulate that something else is involved, in addition to the production of IL-12, IFN- γ , and TNF- α . There are

two logical possibilities. Either immunity requires some additional Th1-associated activity, such as an unidentified macrophage function with variable inter-individual efficacy [463], or immunity mediated by the Th1 response is only effective in the absence of another corrupting influence. Many authors have suggested that this corrupting influence might be a form of Th2-like response (reviewed in [236]), because IL-4 downregulates iNOS [276], drives an inappropriate alternative form of macrophage activation [274] and in animal models [14, 16] can contribute to tissue damage and fibrosis. Moreover, recent human data suggest that those with pre-existing lymphocyte IL-4 responses have a high rate of progression to active TB [15].

However our tenuous understanding of the role of IL-4 production in human tuberculosis is confounded by the fact that earlier studies produced discordant results (discussed later in this chapter) due to technical difficulties in measuring IL-4 (these are outlined in chapter 2, table 2.1). Furthermore, these studies did not distinguish between the agonist IL-4 and its splice variant [33] and antagonist [314], IL-4 δ 2, resulting in data that is difficult to interpret. The two forms can easily be distinguished by RT-PCR. However, even when discriminative nested conventional PCR is used [328] there are a number of drawbacks (outlined in chapter 2 table 2.2) the most important being uncertain validity due to unreliable mRNA quantification. Finally, there are no studies that have examined the compartment-specific (lung versus blood) disparities in IL-4/ IL-4 δ 2 expression in human tuberculosis.

This chapter outlines IL-4 and IL-4 δ 2 gene expression in lung lavage and whole blood, and correlation with clinical parameters, using a validated (chapter 2), reproducible and sensitive real-time PCR assay (chapter 3).

5.2. Methods

Twenty-nine patients with culture positive pulmonary tuberculosis (PTB), 15 of whom underwent lung bronchoalveolar lavage (BAL), were recruited. Control donors (n=21) were healthy volunteers matched to the first 21 sequentially recruited TB patients for age, sex and ethnicity. They were asymptomatic had normal chest radiographs and were selected because of the absence of LTBI despite exposure to *M. tuberculosis* (health care workers and contacts of PTB patients). Six of these

volunteers underwent BAL.

Whole blood (2.5 ml) was taken within the first 2 weeks of anti-TB treatment and immediately transferred from the patient into PAXgene-RNA tubes to fix the mRNA profile. Ten donors with PTB were bled again after 3 months or within 4 weeks of stopping chemotherapy. More detailed participant and experimental details including acquisition of BAL, method of radiographic scoring, TB antigen-specific ELISPOT to exclude LTBI, ELISA for sCD30, details of RT and real-time PCR, and methods relevant to data analysis are outlined in chapter 2 (general methods).

5.3. Results

5.3.1. Clinical and baseline parameters

Demographic details of matched patients and controls (n=21) and BAL cell counts are shown in table 5.1. When all TB patients (n=29) were compared to controls there were no differences in baseline characteristics. None of the study subjects had elevated eosinophil counts and all control patients had normal chest radiographs and were not latently infected with *M. tuberculosis* by antigen specific IFN- γ assay.

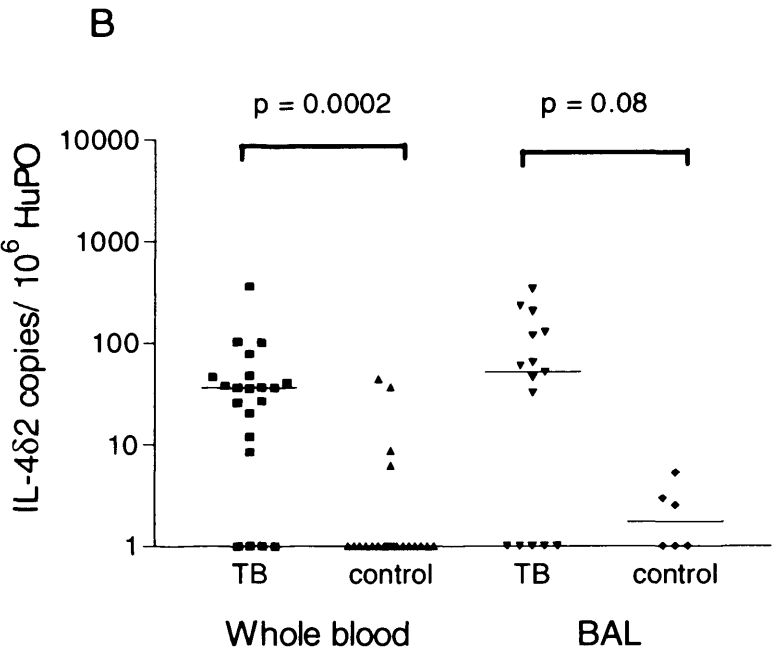
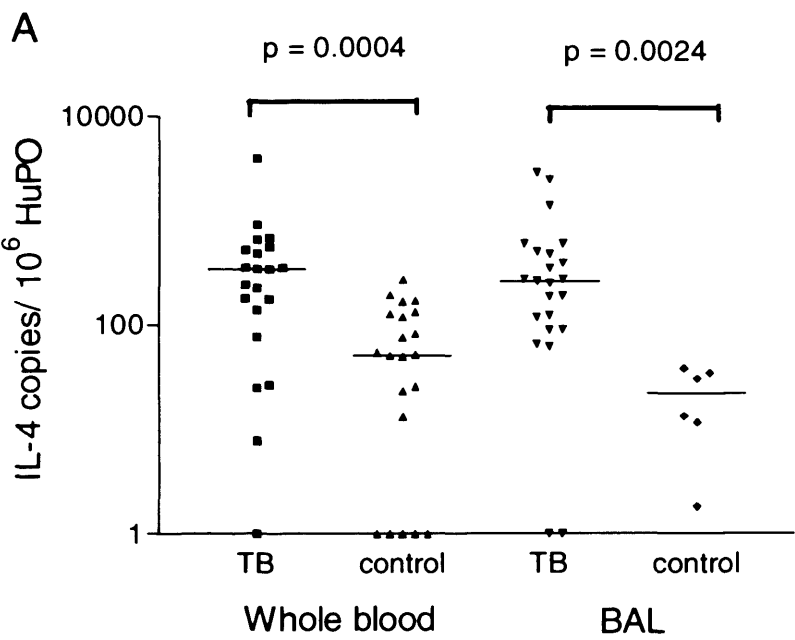
Table 5.1. Demographic details and lavage cell counts at recruitment. Demographic details and radiographic scores are of 21 patients with pulmonary tuberculosis and their matched controls; the lavage cell counts are those of 10 patients and 6 controls.

Numbers in parenthesis = %	TB	Control
Age (median; range in years)	30; 18-81	31; 21-80
Sex (male)	14 (67)	13 (62)
Ethnicity		
Black African	12 (57)	11 (52)
White	4 (19)	4 (19)
Other	5 (24)	6 (29)
X-ray features		
Cavitation	12 (57)	0
>50% airspace shadowing of total lung field	5 (24)	0
BAL (mean \pm SE)		
Total leucocytes ($\times 10^4$ /ml)	28 \pm 19	12.8 \pm 4.2
lymphocyte count ($\times 10^4$ /ml)	308 \pm 106	81 \pm 25
CD4/CD8 ratio	3.58 \pm 0.99	2.55 \pm 0.94

5.3.2. Th2 markers in whole blood and BAL at baseline

Both IL-4 and IL-4 δ 2 mRNA levels (mRNA copies per 10^6 copies HuPO) were significantly elevated in whole blood and lung lavage of PTB vs controls ($p < 0.005$ for IL-4 and IL-4 δ 2, Figure 5.1A and 5.1B); all median and 25th-75th percentiles are shown in table 5.2. Levels of IL-4 and IL-4 δ 2 were not significantly different when whole blood and BAL were compared in paired samples (median mRNA levels 317 vs 118 and 60 vs 45, respectively). The 2 subjects in whom IL-4 was undetectable in alveolar lavage cells had no cavitation and minimal parenchymal lung changes, however, the subjects in whom IL-4 δ 2 was undetectable could not be characterised by disease phenotype. In contrast to IL-4 and IL-4 δ 2, IFN- γ was massively upregulated in BAL ($p = 0.0007$) but not in whole blood (figure 5.1C) of TB donors compared to controls. Furthermore sCD30 was elevated in the plasma ($p = 0.0025$) and BAL fluid ($p = 0.01$, figure 5.2) of PTB vs controls.

In whole blood there was a significant correlation between the PTB total radiographic score and the IL-4/IFN- γ ratio ($p= 0.0034$, Figure 5.3A) and a negative correlation with IFN- γ , $p= 0.005$. In BAL there was a significant correlation between the PTB total radiographic score and sCD30 levels ($p= 0.0001$, Figure 5.3B). Furthermore, TB patients with high levels of cellular inflammation (high BAL total leucocyte count) had high IL-4/IFN- γ ratios ($p= 0.02$ for the correlation).



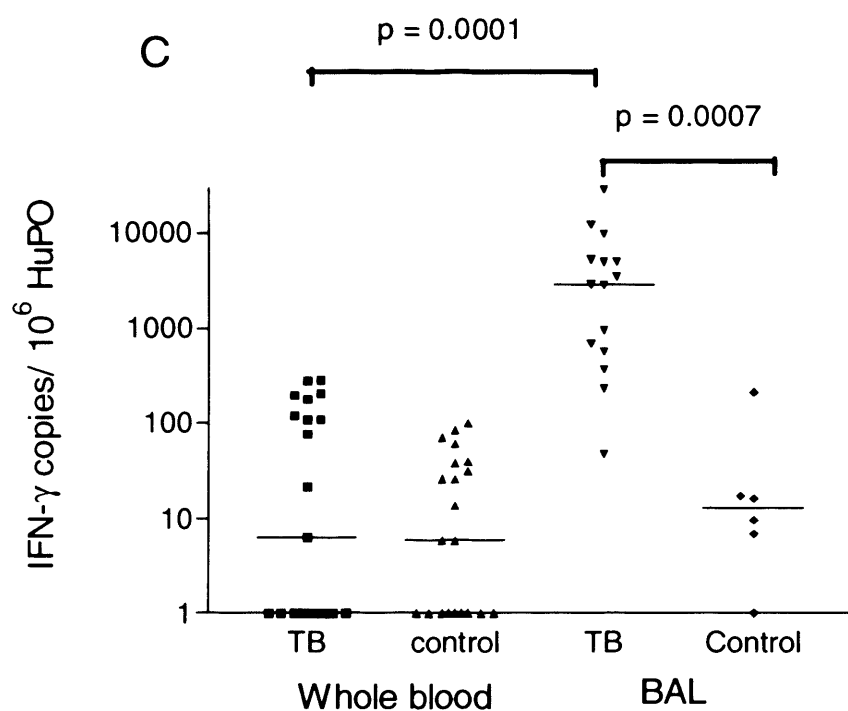


Figure 5.1 A, B and C. IL-4 (A), IL-4 δ 2 (B) and IFN- γ (C) mRNA levels in whole blood and alveolar lavage (BAL) at the start of treatment. Expression of IL-4 and IL-4 δ 2 were significantly increased in patients with tuberculosis compared to controls. IFN- γ was elevated in the lung lavage of PTB vs controls. Cytokine mRNA copy numbers are expressed per million copies of HuPO.

Table 5.2. IL-4, IL-4 δ 2 and IFN- γ values (median; 25th-75th percentiles) in whole blood (n= 21 TB and matched controls), alveolar lavage (n=15 TB and 6 controls) and cell sub-populations of peripheral blood (n=10 TB and matched controls). Also shown are values pre and post anti-TB treatment matched to controls (n=10). All values are expressed as mRNA levels per million copies of HuPO.

	TB	Matched Control	P value
IL-4:			
Whole blood	344; 107-544	52; 7-129	P= 0.0004
BAL	262; 104-551	21; 6-36	P= 0.0024
IL-4 δ 2:			
Whole blood	37; 10-46	1; 1-1	P= 0.0002
BAL	51; 1-166	2; 1-4	P= 0.08
IFN- γ :			
Whole blood	6; 1-148	6; 1-38	P= 0.4
BAL	2858; 627-7458	13; 4-113	P= 0.0007
sCD30 (U/ml):	14.2; 7-22	4.4; 2-6	P=0.003
Plasma	20.3; 0.2-30.4	0.6; 0.1-0.2	P= 0.01
BAL			
Response to Rx:			
IL-4 baseline	348; 25-566	37; 1-143	P= 0.4*
IL-4 post Rx	177; 59-234		P= 0.03**
IL-4 δ 2 baseline	23; 5-43	1; 1-1	P= 0.02*
IL-4 δ 2 post Rx	52; 28-75		P= 0.0002**

* comparison between values at baseline and values after treatment

** comparison between values after treatment and values in matched controls

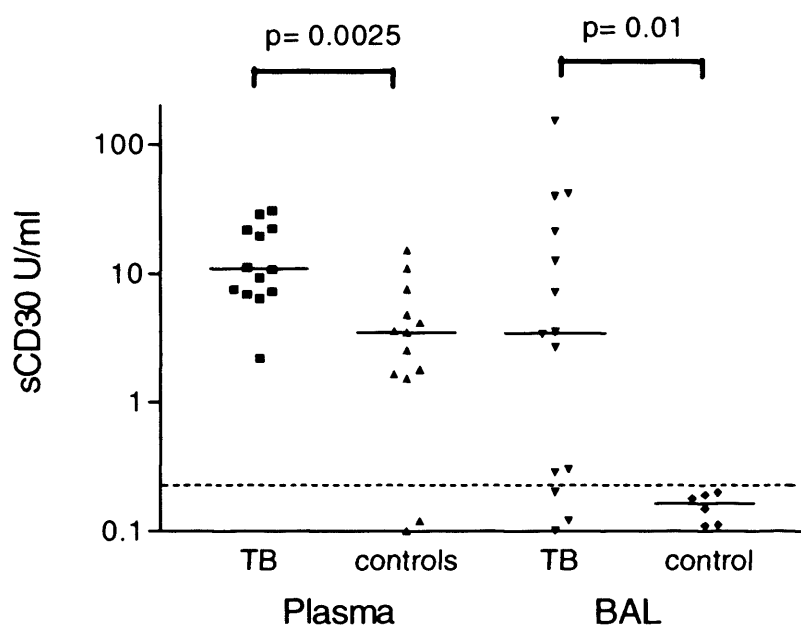
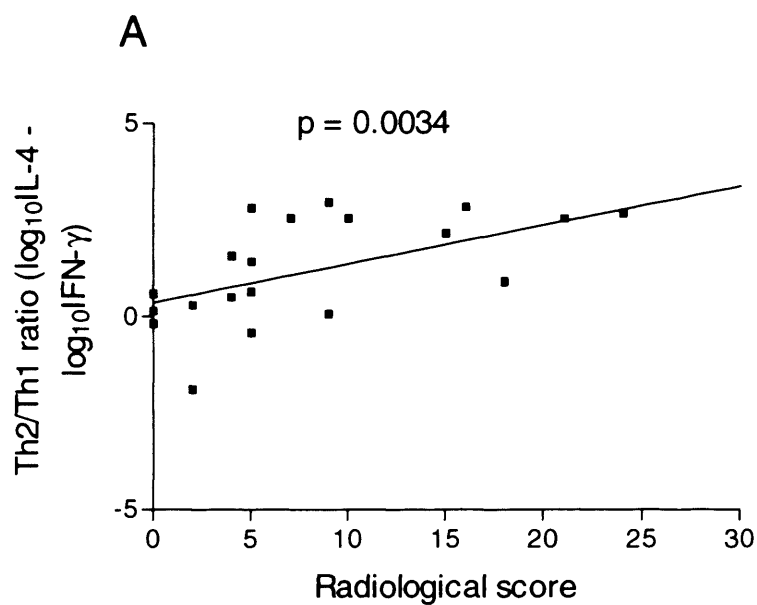


Figure 5.2. Soluble CD30 levels. Soluble CD30 was elevated in plasma (n=13) and alveolar lavage fluid (n=15, alveolar lavage) when patients with tuberculosis were compared to controls (dashed line indicates the detection limit of the assay).



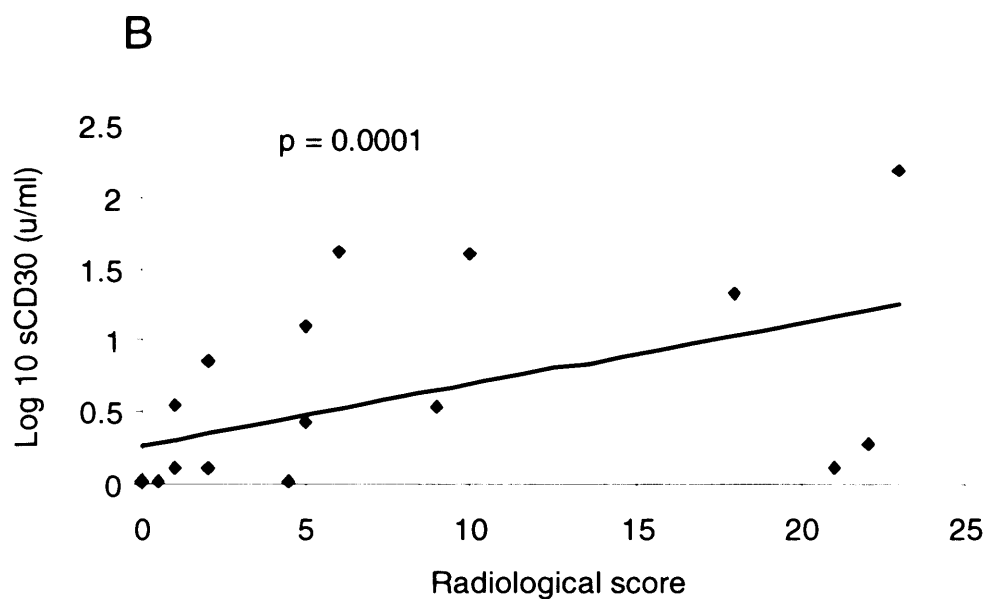


Figure 5.3 A and B. Radiological scores and Th2 markers. There was a significant correlation between the total radiographic score and the whole blood IL-4/IFN- γ ratio (5.3 A, expressed as differences between logged data), and the alveolar lavage sCD30 (5.3 B) in patients with pulmonary tuberculosis.

5.3.3 Cavitory versus non-cavitory disease

When patients with and without cavitation were compared there was no significant difference in the median (25th-75th percentiles) IL-4 mRNA levels in whole blood [310 (158-804) and 354 (211-625), respectively] or BAL [190 (45-438) and 90 (1-219)]. Similarly, IL-4 δ 2 mRNA levels, the Th2/Th1 (IL-4/IFN- γ) ratio and the IL-4/IL-4 δ 2 ratio were similar in both whole blood and BAL.

5.3.4. Comparison of IL-4 relative to IL-4 δ 2 in whole blood and BAL

Although both IL-4 and IL-4 δ 2 mRNA levels were substantially higher in TB compared to control subjects (figure 5.1), the ratio of IL-4/IL-4 δ 2 was not significantly different in the 2 groups (TB and controls) for both whole blood and

BAL.

When only TB subjects were considered and the blood and BAL compartments compared the IL-4/IL-4 δ 2 ratio was not significantly different in 15 paired blood and BAL samples [median (25th- 75th percentiles) ratio of 0.17 (0.0-1.16) and 0.45 (0.06-2.46), respectively].

5.3.3. IL-4 and IL-4 δ 2 in response to anti-TB treatment

After the intensive phase of short course chemotherapy (month 3, n=5) there was no significant change in IL-4 or IL-4 δ 2 mRNA levels (data not shown). However > 6 months after initiation of short course chemotherapy (n=10) there was a non-significant decrease in IL-4 mRNA expression which was still higher than in matched controls (p=0.03, figure 5.4 A and table 5.2). By contrast there was a significant increase in IL-4 δ 2 (p= 0.02, Figure 5.4 B and table 5.2) and IFN- γ mRNA levels (p< 0.0001, table 5.2) after treatment. Furthermore the median IL-4/IL-4 δ 2 ratio and the IL-4/IFN- γ ratio (expressed as log differences between cytokine pairs) significantly decreased > 6 months after initiation of chemotherapy (1 vs 0.4 for the former, p=0.04 and 2.3 vs 0.3 for the latter, p<0.0002).

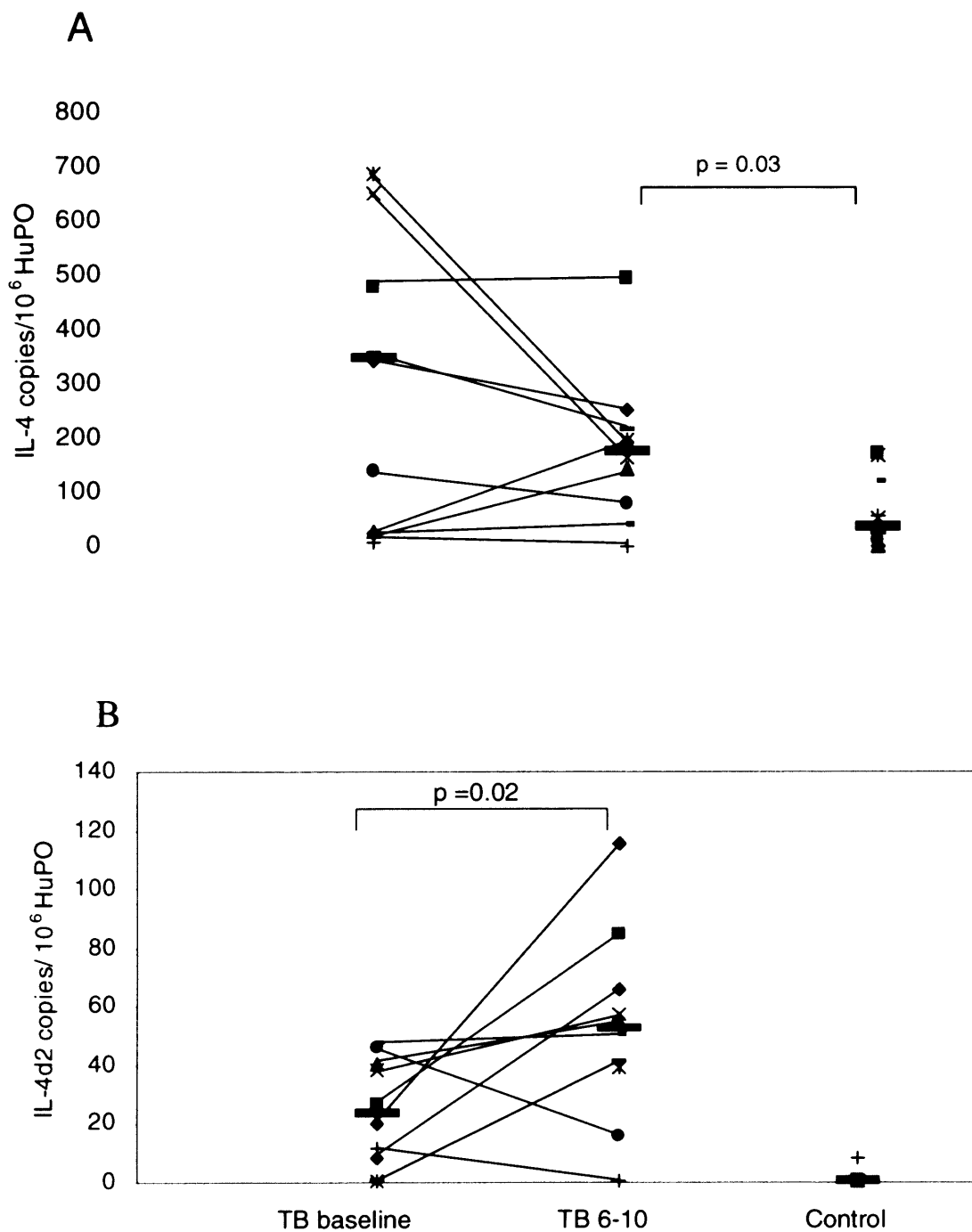


Figure 5.4 A and B. Comparison between IL-4 (5.4 A) and IL-4 δ 2 (5.4 B) mRNA levels at baseline, after 6 months of chemotherapy (TB 6-10) and in matched controls (n=10). At 6 months IL-4 mRNA was non-significantly changed from baseline but significantly higher than matched controls. IL-4 δ 2 at 6 months was significantly higher than at baseline.

5.3.4. ELISPOT assays

PBMC from control donors cultured with ESAT-6 and CFP-10 yielded median (range) IFN- γ spot counts (per million PBMCs) that did not differ significantly from negative control wells (0; 0-52 vs 0; 0-65 spots) suggesting that they were not latently infected.

5.3.5. ELISA for IL-4 and IFN- γ

IL-4 was elevated in the BAL of PTB vs controls (median 12.2 vs 6.9 pg/ml, $p=0.5$) and detectable in 11 of 15 PTB patients and 4 of 6 controls. In plasma, IL-4 levels were not different in PTB vs controls but in most cases levels were around the detection limit of the assay. IFN- γ protein was largely undetectable in BAL or plasma (2 of 15 TB BAL samples and 3 of 13 TB plasma samples).

5.4. Discussion

It has been recognised since the days of Koch that stimulating the acquired immune response may exacerbate the clinical manifestations of TB. However, the pathways that underpin disease progression remain unclear. One hypothesis (others have been discussed in chapter 1, section 1.5) is that a small but significant IL-4 component may subvert the protective Th1 response, while IL-4 δ 2 might be protective (reviewed in detail in 13;30). However, whether IL-4 cytokine levels are raised in TB has been contentious and the data discordant with some studies finding IL-4 [18, 19, 29, 349] and others failing to do so [27, 28, 30, 355]. Furthermore, IL-4 δ 2 mRNA levels and sCD30 have not previously been investigated in BAL fluid of donors with active TB.

In the present study, the first to investigate IL-4 and IL-4 δ 2 mRNA levels using a real-time quantitative assay, found that patients with PTB have significantly greater expression of mRNA encoding both IL-4 and IL-4 δ 2 in whole blood (~ 7 fold and ~37 fold ,respectively) and lung lavage (~12 fold and ~ 26 fold, respectively). Furthermore, pre-treatment radiographic scores correlated with the IL-4/IFN- γ ratio. After short course chemotherapy IL-4 mRNA levels did not decline significantly

whilst IL-4 δ 2 mRNA significantly increased. The implication of these findings and their relationship to other studies is further discussed.

5.4.1. Discordant IL-4 data and findings in relation to other studies

On the one hand, subjects with TB close to the equator may have levels of IL-4 high enough to be detected in the serum by ELISA (discussed later). On the other hand, studies conducted in temperate latitudes require stimulation protocols or PCR to detect IL-4 in donors with active TB [17-19, 186, 345]. Indeed, IL-4 is undetectable by ELISA in Dutch patients with TB [32]. Nevertheless, study results have been discordant due to technical factors related to IL-4 (summarised earlier in table 2.1) and failure to distinguish between IL-4 and IL-4 δ 2, which make most studies relating to IL-4 in TB un-interpretable. Only one study examined IL-4 δ 2 mRNA levels in active TB [17] but interpretation is constrained by the technical limitations of earlier PCR techniques (summarised earlier in table 2.2). To address these problems a probe specific real-time PCR assay was designed (chapter 2) to measure mRNA levels in index cases of tuberculosis. The assay is able to distinguish between IL-4 and IL-4 δ 2 mRNA and circumvents the problems of imprecise quantification, poor reproducibility, low dynamic range of earlier PCR assays. Furthermore, to ensure the detection of small inter-group differences whilst avoiding artefactual changes a validated reference was used to normalise mRNA levels (44) and only quality controlled RNA templates were used. Indeed, probe specific assays have been shown to give better resolution when low copy number genes of interest are studied (46). As IL-4 is expressed at low levels that approach the detection limit of the assay, it is essential that the above considerations are adhered to if meaningful results are to be obtained. This is particularly relevant to the detection of IL-4 in *unstimulated* cells, a strategy used in this study as pre-stimulation protocols are Th1 biased and rapidly turns off IL-4 [35].

The considerations, discussed in the previous paragraph, explain the failure of earlier studies to detect IL-4 [27, 28, 30, 355]. However more recent studies show that IL-4 is detected in antigen-stimulated cells by ELISPOT [18] and flow cytometry [19] and

in unstimulated cells by RT-PCR [17]. Nevertheless, 2 studies from the USA [27, 28] and 2 from developing countries (Mexico and China) [30, 355] failed to detect IL-4 using RT-PCR. The failure to detect IL-4 may be due to delays in fixing the RNA profiles. Indeed, Doherty and co-workers showed that delays of as little as 2 hours may substantially reduce IL-4 detection by ELISPOT and RT-PCR [411]. This may be particularly relevant in Th1 biased *in vitro* cultures where IL-4 is rapidly down-regulated by IFN- γ [27, 28] and the inevitable delays experienced when attempting to fix the mRNA profiles in excised lymph nodes [27]. In contrast to the findings of Lin and colleagues [27], another study detected IL-4 mRNA in tuberculous lymph nodes [464]. Furthermore, in the studies that failed to detect IL-4 details about RNA template quality is not provided and the use of an unvalidated reference (β -actin or GAPDH in all the studies) could have easily obscured significant inter-group differences (proven and discussed in chapter 2). Finally, none of these studies discriminated between the agonist, IL-4, and the antagonist, IL-4 δ 2.

In contrast to studies conducted in temperate countries, those performed within 30 degrees of the equator (except in alpine climates e.g. Addis Ababa) find IL-4 levels that are high enough to be detected in the serum by immunoassays (these studies are listed in table 1.6). This phenomenon may be related to the adjuvant activity of environmental saprophytes or helminths; this is reflected in Th2 responses to BCG in children whose mothers are exposed to parasites [405]. That this effect is purely helminth related is unlikely as matched control subjects from the same areas do not have raised IL-4 levels and upregulated IL-4 responses in TB patients are seen in both Africa and Europe. Nevertheless, epidemiological evidence showing an association between helminth infection and TB is sparse. Elias and co-workers showed an association between active TB and helminth infection in Ethiopia [465] but there were confounding variables that predisposed to both infections and it is uncertain whether helminth infestation was cause or effect. A prospective study of otherwise healthy HIV+ subjects in Africa indicates that eosinophilia, presumably helminth induced, is predictive for progression to active TB [466]. On the other hand, rates of TB are not dissimilar in different parts of Ethiopia where helminth infection is endemic or uncommon, respectively (Dr TM Doherty, SSI, personal communication). Taken together, these data suggest that the high IL-4 levels seen in developing countries are

TB-specific but helminthiasis may simultaneously potentiate Th2 responses by their adjuvant activity. The high background cytokine responses primed by environmental mycobacteria and helminths may also explain the failure of BCG in developing countries and has implications for laboratory testing of vaccine candidates in murine models (discussed in detail in chapter 1, section 1.7.5.2).

5.4.2. IL-4 findings in BAL

In the present study there was a significant ~ 1.5 log increase in IL-4 mRNA expression in TB compared to control donors but IL-4 was largely undetectable in BAL fluid when ELISA was used. There are limited data on IL-4 levels in the BAL fluid of TB patients. Three reports documented raised IL-4 levels [24, 265, 356] whilst IL-4 was undetectable in four studies [264, 357-359]. The latter studies used either immunocytochemistry or in situ hybridisation (relatively insensitive for IL-4) [358, 359], or ELISPOT in PPD stimulated cells [264], or ELISA of lavage fluid [357]. In fact, ELISA was also unhelpful in the present study because of the substantial dilutional effect in BAL (even with 10 fold concentration) and the lack of a satisfactory normalisation strategy. BAL produces a 150 to 300-fold dilution of cytokines [44, 467], resulting in stochastic detection in body fluids. Further concentration of BAL fluid is constrained by technical factors and the very small volumes generated by concentration (< 300µl). The dilutional effect has been documented in other studies using ELISA to measure cytokine levels in BAL fluid [24, 44, 264, 468]. In pulmonary sarcoidosis [469] and systemic sclerosis [44] IFN- γ is undetectable by ELISA but found to be substantially elevated when RT-PCR is used. Consequently, real-time PCR is useful to study IL-4 and IFN- γ cytokine profiles in BAL fluid. ELISPOT is at least one log more sensitive than ELISA [470, 471] but may still fail to detect IL-4, which is physiologically active at concentrations 3 logs below IFN- γ [413, 414]. Finally, cytokine detection is influenced by the characteristics of the segment that is lavaged (presence and degree of inflammation and immunopathology) [468].

In this study there were no detectable differences in the BAL or whole blood IL-4 mRNA levels in subjects with cavitory versus non-cavitory TB. By contrast,

Mazzarella and co-workers, using FACs and ICCS after PMA stimulation, demonstrated that BAL CD4⁺ T cell IL-4 expression was raised in cavitary compared to non-cavitary disease [356]. The use of unstimulated cells in the current study, different degrees of lung involvement, and detection methods used may account for the different findings. Nevertheless, both the current study and two previous reports [17, 19] showed that IL-4 expression in peripheral mononuclear cells correlates with cavitation or the degree of radiographic pulmonary involvement. The data presented in this chapter show that the Th2/ Th1 (IL-4/ IFN- γ) ratio in peripheral blood correlated with radiological disease extent (a composite score of consolidation, reticular infiltrates and cavitation). Furthermore, another surrogate marker of severity, the total BAL leucocyte count (highest in those with greater radiological disease extent), correlated with the IL-4/IFN- γ ratio. Collectively, these data support the contention that IL-4 facilitates human tissue necrosis and immunopathology, as it does in murine models of TB [14, 16].

5.4.3. Comparison of IL-4 vs IL-4 δ 2 expression in BAL and blood

The findings of this study indicate differential cytokine balance in the different body compartments. In subjects with TB the median IFN- γ mRNA level was more than 200 fold higher in alveolar lavage cells than in whole blood. By contrast, the median IL-4 and IL-4 δ 2 mRNA levels were similar in both the body compartments. However, the relationship of IL-4 and IL-4 δ 2 mRNA levels to each other (discussed later) and to the different body compartments was heterogeneous. This may reflect the degree of lung inflammation or disease chronicity in different individuals. When there is inflammation of the alveolus or pulmonary interstitial tissue it is logical to expect some spill over of inflammatory cells and cytokines into the blood compartment. However, the cytokine-specific expression in one body compartment relative to another is poorly understood. It is not size dependent because large molecules like insulin rapidly translocate from the lung into the blood [472]. Furthermore, chemokines like MIP 1- α , but not IL-6, when they are readily detected in the lung of septic patients are poorly detectable in serum [473]. Consequently, cytokine and compartment-specific disparity has to be taken into account to avoid misinterpretation of TB-specific immunological data.

IL-4 δ 2 mRNA levels have not previously been reported in alveolar lavage cells in TB. Atamas and colleagues found that CD8+ alveolar lavage T cells of scleroderma patients, with pulmonary fibrosis and rapidly declining lung function, expressed a mixed IFN- γ / IL-4/ IL-4 δ 2 profile [44]. Furthermore, whilst IL-4 δ 2 is antagonistic to IL-4 in lymphocytes cultures [314], it is an agonist like IL-4, able to stimulate collagen production in human lung fibroblasts [44]. Although IL-4 δ 2 was upregulated in BAL cells in the current study there was no correlation with pre-treatment radiological disease extent (a composite score of lung fibrosis, cavitation and consolidation). Nevertheless, in the early stage of TB there maybe little fibrosis and it would be enlightening to examine the relationship between 6 month radiological scores and the initial IL-4 δ 2 mRNA levels. These data are currently being accumulated as part of a separate study.

5.4.4. The IL-4/ IL-4 δ 2 ratio

The precise function of IL-4 δ 2 remains unknown. However, the available evidence indicates that it serves a regulatory mRNA function (discussed in chapter 4), it acts as a natural antagonist in T cells, B cells and monocytes [314], but is an agonist on fibroblasts [44]. Interestingly, preliminary studies indicate that IL-4 δ 2 mRNA levels are elevated in African subjects with LTBI (as defined by ESAT-6 responses) who remained healthy in the short term [360, 361]. Furthermore, a study of healthcare workers in Portugal has revealed that individuals whose peripheral blood mononuclear cells synthesised IL-4 (IL-4 δ 2 expression was not assessed) in response to *M. tuberculosis in vitro* went on to develop overt TB within 2–4 years [15]. Collectively these data raise the question of whether the ratio between the agonist (IL-4) and the antagonist (IL-4 δ 2) is a correlate of protective immunity. Indeed, the significantly decreased IL-4/ IL-4 δ 2 ratio with anti-TB treatment lends support to this hypothesis. However, against this hypothesis, both IL-4 *and* IL-4 δ 2 mRNA levels were upregulated in TB, and the ratio of the agonist to the antagonist (IL-4/IL-4 δ 2) was similar in patients and controls and in the different body compartments. Nevertheless, it is possible that in the crucial early stages of disease the ratio will be different and favour the agonist, IL-4, thereby facilitating disease progression. Indeed, in BALB/c mice the ratio of IL-4/IL-4 δ 2 changed during the different phases (early

and late progressive infection) of disease (R Hernado-Pandez, personal communication, study in progress). Longitudinal studies will be required to address whether the balance of the agonist (IL-4) and potential antagonist (IL-4 δ 2) might be relevant to disease progression. Crucial unanswered questions are whether IL-4 δ 2 is present only as mRNA or also as active protein, and whether the ratio portends disease progression.

5.4.5. High IFN- γ mRNA levels in BAL compared to whole blood

Over the last decade many reports, using peripheral blood because of the difficulty in obtaining lung specimens, indicated that TB patients compared to healthy volunteers have relatively poor IFN- γ or proliferative responses in peripheral blood [29, 203] and recovery of the IFN- γ response with treatment [77, 474]. This created the notion that vaccines augmenting antigen specific IFN- γ responses therapeutically or prophylactically could prevent or inhibit disease progression. However, our data demonstrate that at the site of disease, the lungs, there are very high IFN- γ mRNA levels (~ 2.5 log increase in IFN- γ mRNA in alveolar lavage cells of TB patients compared to controls but no difference in whole blood). There are only a handful of studies that have used lung cells but their findings are similar. High IFN- γ levels are also found in induced sputum [7], BAL [356] and by RT-PCR in alveolar cells from subjects with active TB [475]. In one of the few studies that compared peripheral blood and lung profiles simultaneously, Schwander and colleagues found that mycobacterial antigen-induced proliferative responses and IFN- γ producing cells were reduced in PBMCs but enhanced in the lung [264].

Collectively, our data and those of other investigators suggest that the idea of a sub-optimal Th1 response in TB is probably incorrect. Of course, a certain minimum requirement for IFN- γ , IL-12 and TNF- α are crucial for protection [114, 153, 343] but further enhancement of Th1 responses are unlikely to be helpful. In addition to the prominence of Th1 cytokines demonstrated here, three other lines of evidence support this view. Firstly, close examination of the tissues in pathological studies of LTBI show minimal cellular infiltration suggesting that little immunological activity is required to maintain this control [51, 137]. Secondly, studies of genetically modified

strains of TB with mutations within transcription factors or associated components (SigH, rpoV and WhiB3) show that relatively few Th1 cells are sufficient to control proliferation, and that the remaining 90% facilitate immunopathology [145, 146]. Thirdly, BCG, a predominantly Th1-inducing vaccine, has poor protective efficacy in countries close to the equator [342]. Enhancing the Th1 response of BCG or sub-unit vaccines does not provide any additional protective efficacy [476-478], in fact, it may exacerbate immunopathology [149]. This was demonstrated a century ago by Robert Koch who showed that tuberculous animals [50] and humans [479] mount a necrotic reaction to components of *M. tuberculosis*. Moreover, animals in which the “The Koch Phenomenon” could be elicited were more susceptible to subsequent challenge than were un-immunised controls [480]. Taken together these data suggest that *M. tuberculosis*, rather than down regulating IFN- γ , actually enhances the Th1 response but at the same time corrupts it, so that it is inefficient at killing bacteria. This is logical as it promotes immunopathology and the continued propagation of *M. tuberculosis*.

The presence of significantly elevated IL-4 mRNA levels in BAL fluid in the presence of high IFN- γ mRNA levels indicates a mixed Th1/Th2 profile. At this point, it is appropriate to clarify that the Th2 hypothesis (IL-4 is deleterious in TB) does not imply a dominant Th2 mediated down-regulation of the Th1 response, but its subversion so that there is disease progression and immunopathology. Indeed, IL-4-mediated immunopathology in mixed Th1/ Th2 lesions have been demonstrated in murine models of TB [14] and in other infectious disease models [369, 391, 481]. These murine data suggest that TNF- α is toxic in Th1-dominant lesions when there is a superimposed Th2 profile. The Th2/Th1 profiles observed in the current study and the correlation of the IL-4/IFN- γ ratio with radiological disease severity suggests that a similar mechanism could be operating in humans. Nevertheless, other murine models (IL-4 or STAT-6 knock-outs) do not support this notion [366, 367]. The latter studies showed a lack of susceptibility to *M. tuberculosis* in IL-4 gene knockout strains C57Bl \times 129SvJ [366] and C57Bl [367] mice, in which IL-4 secretion is *not* characteristic of the infection. In other words these strains of mice, unlike the more relevant BALB/c model, are poor producers of IL-4 to begin with and do not mimic the human situation. Consequently, IL-4 gene disruption may not have had

appreciable effects on measures of susceptibility. Indeed, Huygen and co-workers showed that the type of immune response (Th1 vs mixed Th1/ Th2) to mycobacterial antigens is dependent of the strain of mice (C57BL vs BALB/c) [482].

5.4.6. Changes in IFN- γ and IL-4 mRNA levels with anti-TB treatment

This study found that whole blood IFN- γ mRNA levels were similar to control subjects at diagnosis and increased significantly after 6 months of anti-TB treatment. Relatively few studies have analysed cytokine levels or responses during the course of anti-TB treatment. However, similar to the findings presented in this study, Torres and co-workers found that PBMC IFN- γ mRNA levels (and antigen induced proliferation) were enhanced by the end of treatment [30]. By contrast, at the site of disease, IFN- γ levels in induced sputum decrease with progressive treatment [7]. Collectively, these data suggest that cytokine levels during the course of treatment are also compartment-specific. In the current study it would have been interesting to investigate BAL IFN- γ mRNA levels post-treatment but this was not possible due to ethical constraints. A few studies have also investigated antigen-specific IFN- γ responses of PBMCs during the course of anti-TB treatment. IFN- γ responses to the 30kDa antigen [30], PPD [474] and other specific antigens [483] were enhanced after treatment. Similarly, four studies found that ESAT-6 specific IFN- γ responses increased during the course of treatment. By contrast, Pathan and Carrara [484, 485] and my own unpublished observations have indicated that ESAT-6-specific responses declined during treatment. Two conclusions are evident from these discordant findings. Firstly, responses (increase or decline) during the course of treatment may be antigen specific. Secondly, for a particular antigen the results may depend on the formulation used (protein vs peptides), the detection method (ELISA vs ELISPOT) and the incubation time (probably reflects effector vs memory responses; discussed in detail in chapter 1, section 1.3).

After short course chemotherapy the median IL-4 mRNA level, though lower, was not significantly different compared to pre-treatment levels (in five patients the levels remained the same or increased and in 5 patients the levels declined) but significantly higher than in control subjects. This is similar to the findings of Seah et al, who found that IL-4 mRNA levels were not significantly different in whole blood after 3 months

of anti-TB treatment [349]. The failure of IL-4 to decline substantially despite clinical, radiological, and immunological improvement (IFN- γ levels increased significantly in all patients) argues against IL-4 being a non-specific inflammatory bystander response. Rather, if IL-4 antagonises mycobactericidal macrophage activity it may explain why even after several months of treatment, when bacterial load is extremely low, the immune response still cannot contain the disease, and treatment must continue. Alternatively, IL-4 mRNA levels may take considerably longer to decline; it is unclear if they do.

Nevertheless, IL-4 δ 2 levels, not previously studied in response to chemotherapy, increased significantly during the course of treatment. Given the pattern of IL-4 treatment-associated change, this increase in IL-4 δ 2 is unlikely to be due to a reversal of the peripheral immuno-suppressive effect of TB. These findings are relevant to the study of surrogate immunological and serological markers denoting successful treatment and cure of TB [486] and are a priority for the evaluation of new therapeutic vaccines and anti-TB drugs. There were two patients in whom IL-4 δ 2 levels did not increase at the time of assay; both had disseminated disease, and it is possible that the rise in IL-4 δ 2 was delayed in those patients. Further studies are required to clarify the role of IL-4 δ 2 in TB infection, longitudinal changes during active disease and whether IL-4 δ 2 is a correlate of protective immunity. IL-4 δ 2 illustrates the underlying complexity of the Th2 response in TB and this may explain the difficulty in categorising TB into the classical Th1/Th2 paradigm.

5.4.7. IL-4 mRNA levels and biological relevance

The real-time PCR data in this study reflect steady state mRNA levels only. They convey no information about mRNA transcription rates, mRNA stability, or protein levels. Nevertheless, PCR was chosen as it is the only technique able to discriminate between IL-4 and IL-4 δ 2, and that is sensitive enough to detect IL-4 in *unstimulated* cells (these considerations and comparison with other techniques have been discussed in detail in chapter 2, section 2.1.1). Measurement of IL-4 protein levels by ELISA were attempted in plasma, BAL fluid, culture supernatants (chapter 7) and by ELISPOT in cell sub-populations (outlined in chapter 6). However, detection of IL-4

was limited by the poor assay sensitivity, rapid cellular uptake, binding to sIL-4R and the dilutional effect in BAL fluid. Moreover, in samples where IL-4 was detected it remains unclear whether the antibodies used bind IL-4, IL-4δ2, or both proteins.

Despite these limitations the correlation of the IL-4/IFN- γ ratio with radiological disease extent suggest that these results are biologically meaningful. Other investigators have also shown a correlation between IL-4 and radiological patterns [17, 19]. The raised IL-4 mRNA levels are also validated by the significantly elevated levels of plasma and BAL sCD30, which also correlated with radiological disease extent (despite the very high IFN- γ level in the lungs). Although expression of sCD30 is not confined to Th2 cells there are quantitative differences in cellular expression such that sCD30 correlates well with Th2-based markers in asthma, seasonal allergy and atopic dermatitis (reviewed in [333]). The functional relationship between sCD30 and membrane CD30 is uncertain. A putative mechanism by which IL-4 mediated expression of CD30 modulates immune responses is by promoting TNF mediated lymphocyte apoptosis, which may facilitate immunopathology [26]. Recent work has suggested that CD30 is expressed on CD25+ regulatory T cells that can suppress the activity of CD8+ T cells by causing them to undergo apoptosis [487]. A regulatory role that decreases T cell function is compatible with the data presented here.

5.4.8. Selection of patients and controls

As immunological profiles may vary with the disease site only patients with culture positive pulmonary tuberculosis were recruited. To avoid treatment related effects, whilst at the same time allowing practical collection of samples, all specimens were taken before or within the first 2 weeks of anti-TB treatment. Nevertheless, it is difficult to control for inter-individual variations in disease chronicity at the time of diagnosis and differing bacterial loads.

To determine the significance of biological markers in active TB (correlate of susceptibility or epiphenomenon) the ideal comparator group is one, which is 'immune' to *M. tuberculosis* and free of infection (it is now clear that LTBI is not immunologically silent [75]). Until recently, the TST provided the only way to select out those who had been exposed but remained healthy. However, this TST based

distinction was 'muddy' because it could not discriminate between subjects with false positive reactions due to BCG and environmental mycobacteria, and those with LTBI, some of whom may progress to TB over the short term. Ideally, it would have been instructive to study a control group with LTBI simultaneously with one that was exposed but not latently infected. However, in the present study subjects with LTBI were excluded due to resource constraints and as they were already being studied as a cohort in a related study (VACSYS/ VACSEL). Furthermore, we viewed exposed but not latently infected controls as a group 'resistant' to TB infection, and as such constituted an appropriate control group compared to 'susceptible' subjects that developed progressive disease. Samples from subjects with LTBI and donors with other respiratory diseases (including sarcoidosis) have been stored for future analysis.

5.5. Conclusion

The data presented here, using an accurate real-time quantitative assay, indicate that IL-4 and IL-4δ2 mRNA levels are significantly elevated in donors with TB, compared to healthy controls, both in whole blood and the site of disease. These data add to the accumulating evidence that a small but significant Th2-like response is indeed present in tuberculosis. The biological significance of these results is supported by the analogous expression of sCD30 and correlation with immunopathology (radiological scores). These findings are compatible with a hypothesis that IL-4 may subvert protective host mechanisms. The patterns of Th2 cytokine gene expression with anti-TB treatment suggest that IL-4 is not an inflammatory epiphenomenon; the cause-effect relationship is explored further in chapter 7. The usefulness of IL-4δ2 or the IL-4δ2/IL-4 ratio as longitudinal markers of disease activity deserve further investigation. The differential expression of IFN-γ in blood and BAL indicate that cytokine profiles may be compartment-specific and that tuberculosis progresses despite the prominence of a supposedly protective Th1 response.

CHAPTER 6: PERIPHERAL BLOOD CELL SUB-POPULATIONS MAKING IL-4 AND IL-4δ2

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6.1. Introduction

The previous chapter outlined that IL-4 and IL-4δ2 mRNA levels are significantly elevated in active pulmonary tuberculosis and the IL-4/IFN- γ ratio correlates with pre-treatment radiological disease scores (chapter 5). However, there are few data about the cell sub-populations in whole blood expressing IL-4. Moreover, there are no data regarding the T cell sub-types that express IL-4δ2 in human tuberculosis.

There are few data on the contribution of cell types other than lymphocytes to TB-associated immunopathology. Murine and human pathological studies indicate that neutrophils may be important for granuloma formation and tissue necrosis [111, 123]. BAL neutrophil counts are significantly higher when obtained from lung segments affected by cavitation [356, 357] and IL-4 is associated with immunopathology in human [17, 19, 356] and animal models of TB [14, 16]. However, there are no human data on the contribution of non-T cells to IL-4 production in TB.

To gain further insight into the nature of the IL-4 response in human tuberculosis IL-4 and IL-4δ2 mRNA expression was investigated by real-time RT-PCR and ELISPOT in sub-populations of peripheral blood (PBMCs, total T cells, CD4 and CD8⁺ T cells and a population depleted of T cells) in ten patients with PTB and ten controls that were not latently infected. The relevant laboratory and experimental methods have been outlined in chapter 3.

6.2. Results

6.2.1. Purity of separated cell fractions

Mean cell purities of the relative whole blood fractions were > 95%, 99%, 93% and 90% for T, total non-T, CD4+ and CD8+ fractions, respectively. Flow cytometry dot plots of the CD3+ T-cells and non-T cell fractions are shown in figure 6.1.

6.2.2. T and total non-T sub-populations making mRNA encoding IL-4 and IL-4δ2

The IL-4 and IL-4δ2 mRNA levels in CD3+ T cell fractions were significantly higher in PTB than in controls ($p=0.01$ for IL-4 and $p=0.02$ for IL-4δ2, figure 6.2 A and B, respectively; median and 25th-75th percentile mRNA levels for all cytokines are shown in table 6.1). Patients with PTB had higher absolute levels of IL-4 and IL-4δ2 mRNA in both T cells (IL-4 ~ 25 fold and IL-4δ2 ~ 100 fold greater) and non-T cells (IL-4 ~ 6 fold and IL-4δ2 ~ 100 fold greater). For a given production by non-T cells, the T cells of TB patients produced significantly more IL-4 than controls ($p=0.02$, figure 6.3); T cells contained 25% of the IL-4 mRNA in TB.

By contrast, in healthy exposed volunteers both IL-4 and IL-4δ2 were produced mainly by non-T cells compared to T cells ($p<0.05$, figure 6.2 A and B); 93% of IL-4 mRNA in control patients was made by non T cells. Furthermore, as the T-cell fraction was ~ 95% pure it is possible that the expression of IL-4 by T-cells of controls is even lower.

6.2.3. CD4 and CD8 sub-populations making mRNA encoding IL-4 and IL-4δ2

The increase in IL-4 mRNA in T cells from patients with PTB was present both in CD4+ T cells (IL-4 mRNA ~ 7 fold greater in TB) and CD8+ T cells (IL-4 mRNA ~ 10 fold greater in TB). However, the increase in IL-4δ2 mRNA in T cells from TB patients was confined to CD4 T cells (~ 500 fold more than in controls). There was no difference in IL-4δ2 expression in the CD8+ T cells from PTB patients and controls.

6.2.4. The Th2/ Th1 and IL-4/IL-4 δ 2 ratio

The Th2/Th1 (IL-4/IFN- γ) ratio was significantly higher in the T cells of TB patients compared to control subjects (-0.7 vs -10.1; $p = 0.02$). Furthermore, in TB there was a trend in patients with a higher IL-4/IFN- γ ratio having greater radiological disease extent scores ($p = 0.06$; figure 6.4). In TB and control subjects the ratio of IL-4 to IL-4 δ 2 was not significantly different in the various cell sub-populations.

6.2.5. ELISPOT assays

ELISPOT assays were performed to confirm the production of IL-4 and/or IL-4 δ 2 protein. It is not known whether the ELISPOT assay detects one or both of these cytokines. Significant numbers of IL-4/IL-4 δ 2 spots were detected in 90% of PBMC, 60% of non-T cells, 92% of T-cells, 92% of CD4+ T cells and 92% of CD8+ T cells where either cytokine was detectable by PCR. In the PBMC fraction of one TB patient IL-4 was detected by ELISPOT but not by RT-PCR. However all other cell fractions of this patient had IL-4/ IL-4 δ 2 detectable by both methods. There was no correlation between the magnitudes of IL-4/IL-4 δ 2 responses between the two methods, but these findings confirm that IL-4 mRNA correlates with production of active protein.

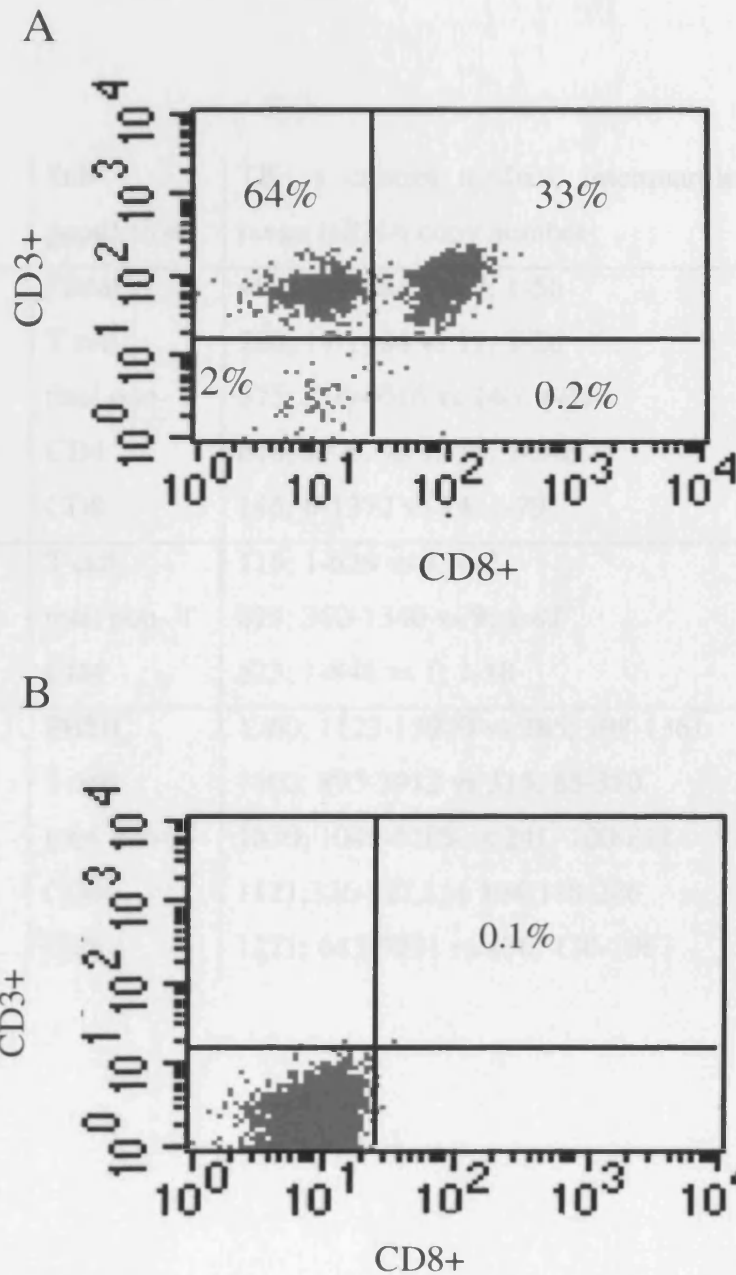


Figure 6.1 A and B. Flow cytometry dot plots of the CD3+ T-cells (6.1 A) and non-T cell fractions (6.1 B) indicating purities of 97% and 99%, respectively (cells were stained with anti CD8-PE and anti CD3-PercP antibodies, respectively)

Table 6.1. Cell sub-populations of whole blood and expression of mRNA for IL-4, IL-4 δ 2 and IFN- γ . All values represent comparisons (median; 25th-75th percentiles) between TB and controls.

Cytokine	Sub-population	TB vs control median; interquartile range mRNA copy number	P value
IL-4 mRNA	PBMC	480; 162-4867 vs 7; 1-56	P< 0.05
	T cell	280; 17-1484 vs 11; 1-26	P= 0.01
	total non- T	875; 336-4016 vs 140; 4-314	P= 0.005
	CD4	616; 37-6299 vs 11; 1-248	P= 0.04
	CD8	146; 6-1372 vs 14; 1-79	P< 0.05
IL-4 δ 2	T cell	115; 1-629 vs 1; 1-2	P= 0.02
	total non- T	874; 360-1340 vs 9; 1-82	P= 0.06
	CD4	523; 1-848 vs 1; 1-18	P= 0.06
IFN- γ	PBMC	1260; 1123-13970 vs 285; 108-1361	P= 0.04
	T cell	1402; 893-3912 vs 315; 85-380	P= 0.002
	total non- T	1670; 1049-5108 vs 241; 100-811	P= 0.04
	CD4	1121;326-3212 vs 194;118-286	P= 0.002
	CD8	1271; 643-9291 vs 430; 130-1067	P= 0.04

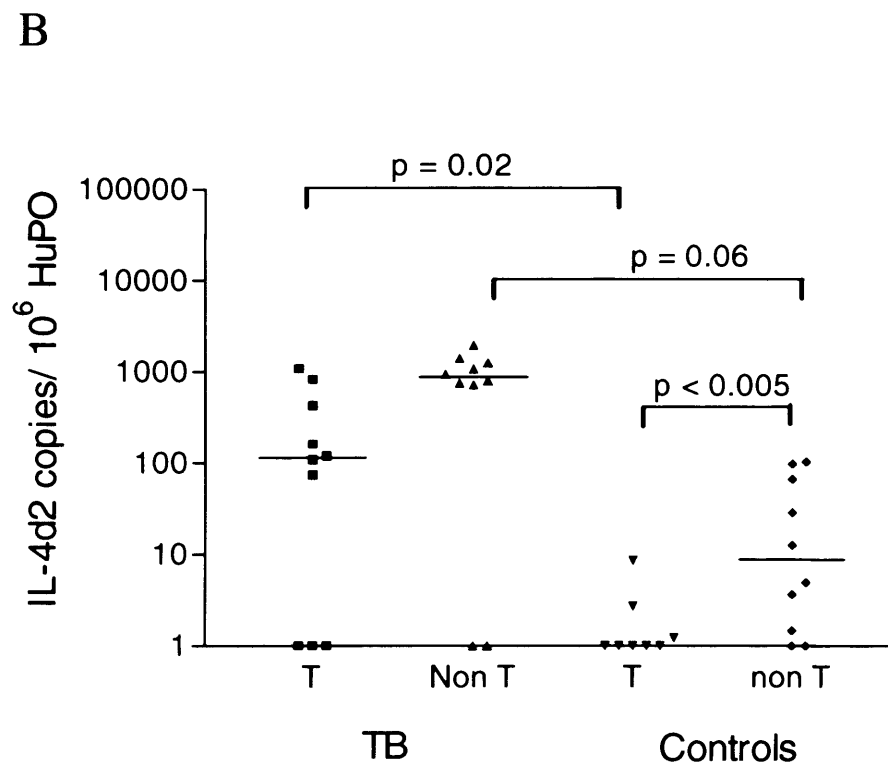
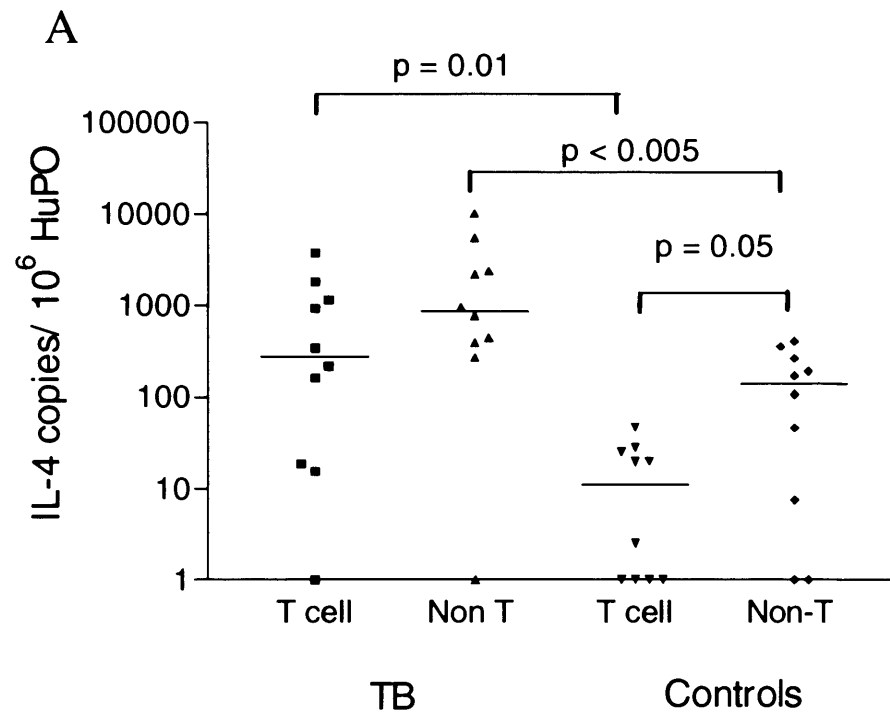


Figure 6.2 A and B. IL-4 (6.2 A) and IL-4δ2 (6.2 B) mRNA levels in CD3+ T cell and total non-T cell fractions. IL-4 and IL-4δ2 levels were significantly higher in the T cells and non-T cells of PTB vs controls.

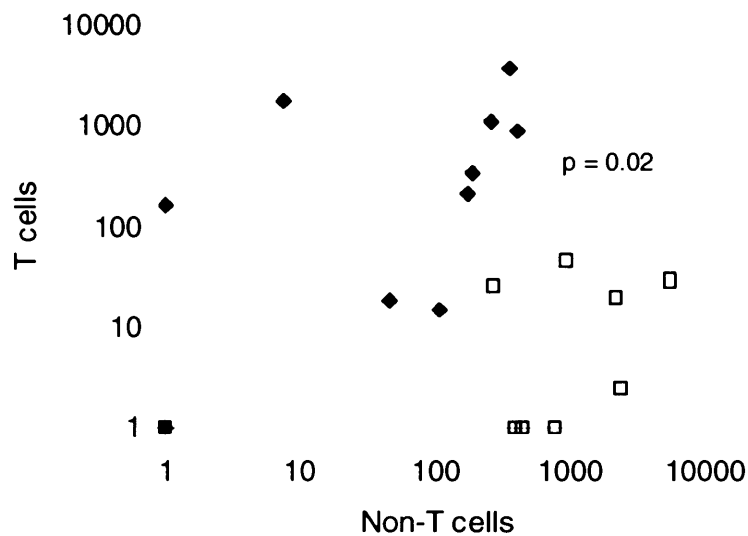


Figure 6.3. Relationship between T and non-T cells for IL-4 production. For a given production by non-T cells the T-cells of TB patients (diamonds) produced significantly more IL-4 than controls (squares).

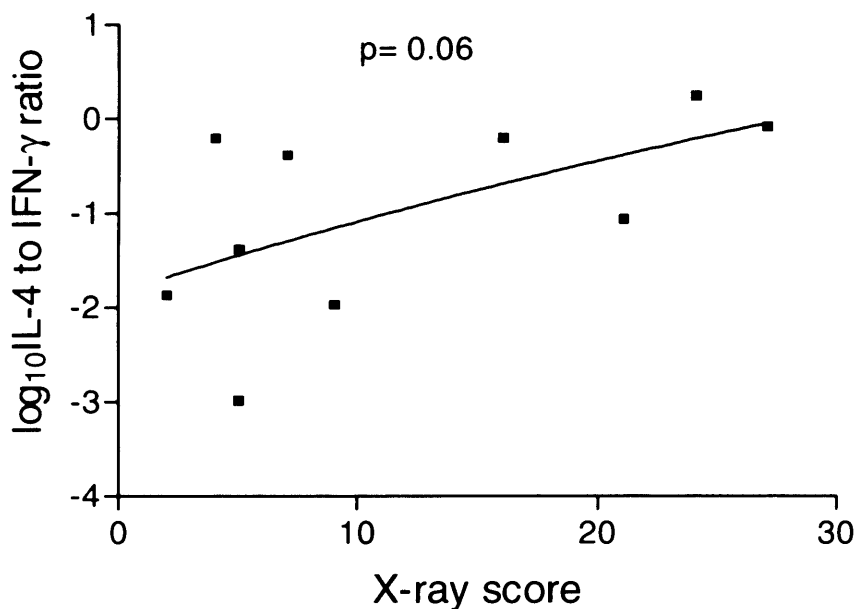


Figure 6.4. Relationship between the peripheral T cell IL-4/IFN-γ ratio and x-ray scores in 10 subjects with pulmonary tuberculosis.

6.3. Discussion

6.3.1. IL-4 mRNA levels and their significance

As already outlined in chapter 1 and chapter 5, Th2 cytokines are causally associated with disease progression and immunopathology in animal models [14, 16]. However, this relationship is more difficult to prove in humans. In chapter 5, the change in mRNA levels with chemotherapy implying that IL-4 is less likely to be a inflammatory bystander effect, was discussed. The data presented here demonstrate that the production of IL-4 and IL-4 δ 2 is predominantly T cell mediated (~ 25-fold increase) suggesting that this is a true antigen driven response. Previously described phenomena, like TB-specific IgE [351] and antigen driven T cell clones producing IL-4, support this contention [348]. Furthermore, evidence suggests that activation of CD8+ T cells during polyclonal activation is antigen specific with minimal bystander activation of T cells [488]. The biological relevance of these results and association between pathology and IL-4 expression is supported by the correlation of the T cell Th2/Th1 (IL-4/IFN- γ) ratio with the extent of radiological disease.

Our findings concur with those of van Crevel, who found that CD8+ T cells make IL-4 in TB and this correlates with cavitation [19]. Other investigators using flow cytometry and ICCS in TB antigen stimulated lymphocytes also found raised IL-4 in CD8+ [29] and CD4+ T cells [21, 347]. However, two older studies (1995 and 1997) failed to find raised IL-4 mRNA in stimulated CD4 T cells [28, 355]. The likely reasons and limitation of these studies have already been discussed (chapter 5, section 5.4.1). Nonetheless, the reagents used in these studies do not distinguish between agonist and the antagonist raising the likelihood that the cells making IL-4 in these reports might be really making IL-4 δ 2.

Although T cells produce significant amounts of IL-4 and IL-4 δ 2 in PTB, it is unclear whether these are from effector or Th2 derived regulatory T cells or both. Although not conclusive, the increasing whole blood and T cell IL-4 response seen with more extensive pulmonary involvement in this study and others (22;33) argues

against regulatory T-cells being the primary source of IL-4.

6.3.2. IL-4δ2 mRNA levels

Whilst IL-4 gene expression has been demonstrated in CD4+ and CD8+ T-cells, the production of IL-4δ2 has not previously been taken into account. Moreover, the antibodies used for flow cytometric studies do not distinguish between the agonist and the antagonist. The current data show that, compared to controls, IL-4δ2 mRNA levels are significantly increased in the T cells of patient with TB. However, in both patients and controls there was no difference in the IL-4/IL-4δ2 ratio in CD4+ or CD8+ T cells. Does this imply, unlike the data generated in latently infected contacts, that the balance between the agonist (IL-4) and antagonist (IL-4δ2) is irrelevant to disease progression and immunopathology? The IL-4/IL-4δ2 ratio only reflects an ex-vivo 'snapshot' and does not reflect profiles in the earliest stages of disease. Indeed, Ordway and colleagues found that asymptomatic subjects with pre-existing IL-4 responses had a high rate of progression to active disease [15]. Nevertheless, how much of this 'IL-4' was IL-4δ2 is unclear [15]. It is intriguing to speculate that the IL-4/IL-4δ2 ratio changes with disease progression and may represent a potential correlate of susceptibility and progression. Although it was not logistically possible, it would have been instructive to repeat the cell subpopulation experiments at the end of anti-TB treatment.

When TB and control patients were compared the IL-4δ2 mRNA levels were upregulated in CD4+ T cells (~ 500 fold) but not significantly different in CD8+ T cells. By contrast, Atamas and colleagues found that CD8+ alveolar lavage cells were the dominant cell type expressing IL-4 in scleroderma patients with rapidly progressive pulmonary fibrosis [44]. Interestingly, in several patients CD8+ T cells expressed both IL-4 and IL-4δ2 mRNA, though the latter did not correlate with lung function or the degree of alveolitis. IL-4δ2 is antagonistic to IL-4 with regard to lymphocyte function [314, 324], but it is an agonist on fibroblasts and drives collagen production [44]. Whether IL-4δ2 antagonises IL-4 and reduces inflammation, or agonises with IL-4 to drive lung immunopathology, remains unclear and needs further investigation.

6.3.3. Expression of Th2 cytokines in the total non-T cell fraction

Notably, in TB patients the total non-T cell fraction (granulocytes and NK cells) also produced significantly more IL-4 than control subjects (here > 90 % of IL-4 mRNA was produced by the non-T cell fraction). To what extent granulocytes facilitate disease progression and Th2 production in TB is unclear. It was recently demonstrated that eosinophil infiltration and degranulation occurs in the earliest necrotic lesions of guinea pig tuberculosis (about 5 to 10 days) [489] and eosinophils have also been demonstrated in human tuberculosis lesions [490, 491]. Eosinophils facilitate antibody-dependent cellular cytotoxicity, chemokine receptor expression, cellular influx into the respiratory tract, and interact closely with Th2 CD4⁺ T cells in mediating cytokine production [492]. Similarly, neutrophils may also facilitate tissue necrosis [111, 123, 124] challenging the traditional dogma that CMI-mediated macrophage destruction underpins caseous necrosis (discussed in detail in section 1.4.2). Neutrophils have also been implicated in early protective immunity to tuberculosis [493]. Other cell sub-types like tissue mast cells, which produce IL-4, can induce immunological responses by directly interacting with *M. tuberculosis* [494]. Nevertheless, the significance of mast cells and granulocytes, their role in Th2 cytokine production, and how they interact with T cells remains unclear, and requires further investigation.

6.3.4. IL-4 detection by ELISPOT compared to real-time PCR

The lack of correlation between the magnitude of IL-4 detected by ELISPOT and real-time PCR is consistent with other reports [34] and may reflect the different sensitivities of the assays, the fact that the ELISPOT may have measured both IL-4 and IL-4 δ 2 simultaneously, and dichotomy between mRNA and protein levels. Furthermore, ELISPOT detects the number of cells secreting the cytokine, not the total quantity of cytokine produced. Although ELISPOT may detect as little as one cell per million PBMCs pre-stimulation is usually required (discussed in chapter 5) and it may not be as sensitive as real-time PCR where mRNA is amplified ~ a million fold. Nevertheless, it has been demonstrated in this study that IL-4 (and/ or possibly IL-4 δ 2) is produced at protein level. Moreover, the demonstrated association between the

T cell cytokine profile and radiologically detected pathology suggests that the results are biologically meaningful.

6.4. Conclusion

IL-4 and IL-4 δ 2 mRNA levels have not previously been investigated in T compared to non-T cell sub-populations in whole blood of donors with tuberculosis. Furthermore, the type of "IL-4" expressed in these cells required clarification. The predominant T cell production of IL-4 suggests that it is a true antigen driven response rather than bystander production by other cell types. Nevertheless, the role of other effector cell types (Treg, NK T cells, $\gamma\delta$ T cells) in producing IL-4 require further clarification. The production of IL-4 by non-T cells requires further investigation particularly in relation to immunopathology. Finally, the role of IL-4 δ 2 and the significance of the IL-4/IL-4 δ 2 ratio in tipping the balance in favour of disease progression needs further investigation.

CHAPTER 7: IL-4 AND IL-4δ2 mRNA LEVELS IN TB ANTIGEN DRIVEN CULTURES.

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7.1. Introduction

Studies investigating cytokine profiles, including IL-4, generated by antigen driven cultures from TB patients are limited and are discussed later in relation to the data presented in this chapter. In TB both IL-4 and IL-4δ2 increase in parallel (chapter 5) compared to bronchial asthma where IL-4 is the dominant form, being expressed at 1000 fold greater than IL-4δ2 [331]. Findings in contacts with LTBI and the fact that IL-4 and IL-4δ2 are physiologically antagonistic raises the possibility that the balance between the two may determine susceptibility and progression in TB. If this hypothesis is correct then it would have implications for vaccine design and developing surrogate markers for protective immunity. However, the relationship between IL-4 and IL-4δ2 in antigen driven cultures from TB patients has not previously been investigated. To study this question, the PBMCs of eight patients with PTB (median age 45 years; 5 Black Africans and 3 Caucasians) and four healthy volunteers (median age 32; 1 Afro-Caribbean and 3 Caucasians) were incubated with sonicated TB antigen for several days. IL-4 and IL-4δ2 mRNA levels were measured by real-time PCR; more detailed methods relevant to this chapter are outlined in chapter 3.

7.2. Results

7.2.1. IL-4 and IL-4 δ 2 expression in PBMC culture

Sonicated TB antigen preferentially induced the expression of IL-4 rather than IL-4 δ 2 when PBMCs from 8 TB patients were stimulated over 7 days (Figure 7.1 A and B). When cells stimulated with TB sonicate were harvested on days 1 or 2, expression of IL-4 was increased compared to baseline ($p= 0.02$), and compared to unstimulated wells ($p= 0.0006$), and to wells stimulated with *M. vaccae* sonicate ($p= 0.03$).

When harvested on days 3 or 4 expression of IL-4 was increased compared to unstimulated wells ($p= 0.02$). Median (range) mRNA levels are shown in table 7.1. A similar pattern was observed using cells from 4 healthy normal volunteers though the median IL-4 mRNA levels were 5 fold lower than in the TB patients' cells and not significantly increased compared to unstimulated wells (figure 7.2).

IL-4 was largely undetectable by ELISA in culture supernatants. At all time points IFN- γ expression was not significantly different in TB patients compared to control donors.

7.3.2. Proliferative responses

In TB patients proliferative responses (expressed as a stimulation index relative to the negative control) to both TB sonicate and *M. vaccae* sonicate were variable and not significantly greater than in unstimulated wells but significantly lower than in PHA stimulated wells ($p< 0.0002$; table 7.2).

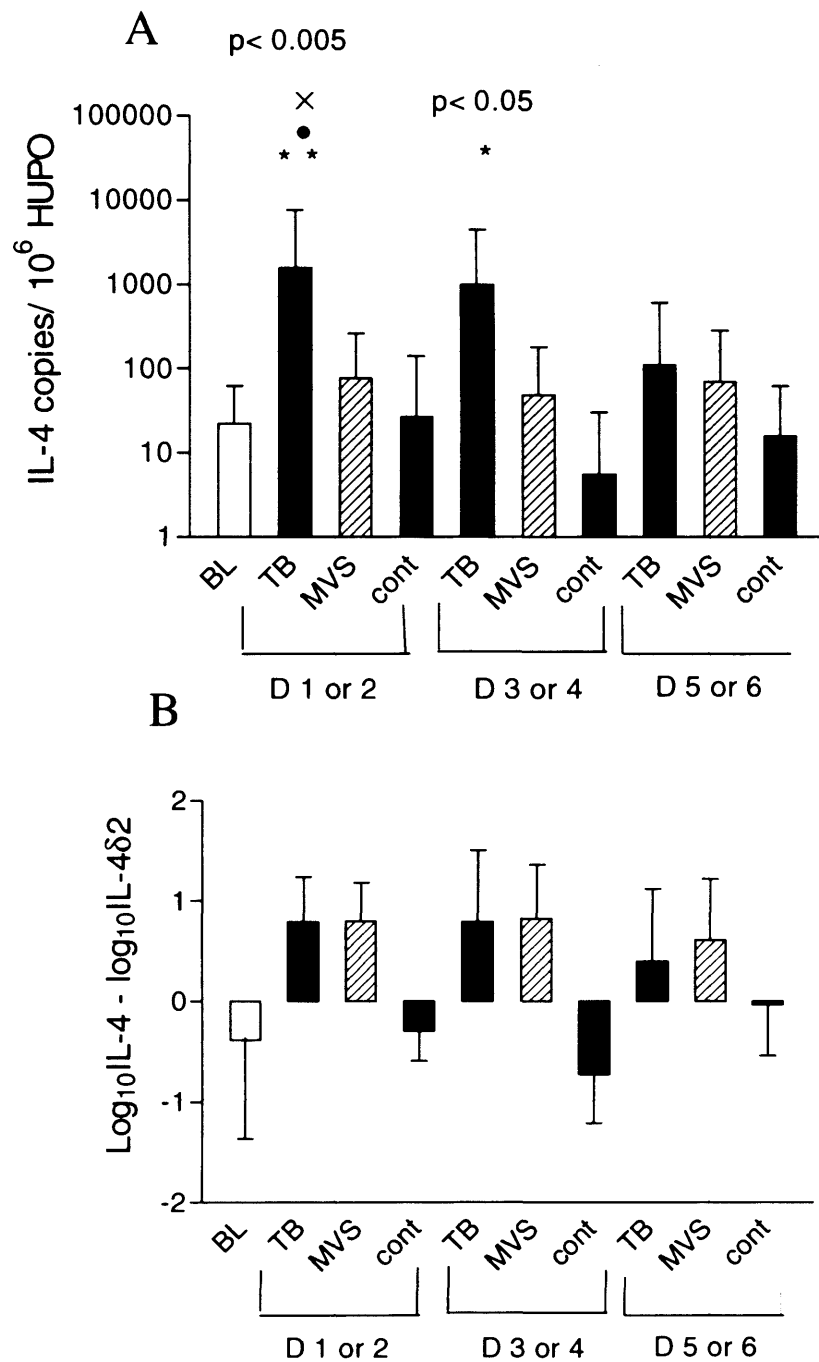


Figure 7.1 A and B. Cultures of blood mononuclear cells from 8 TB patients. Sonicated TB antigen drives the expression of IL-4 (7.1 A) relative to IL-4 δ 2 (7.1 B, ratio of \log_{10} IL-4 to \log_{10} IL-4 δ 2 normalised to HUPO). When stimulated cells were harvested on days 1 or 2 (D 1 or 2), expression of IL-4 was increased compared to baseline [$p < 0.05$ (*)], and compared to unstimulated wells [$p < 0.005$ (**)], and to wells stimulated with *M. vaccae* sonicate [$p < 0.05$ (x)]. When harvested on days 3 or 4 expression of IL-4 was increased compared to unstimulated wells [$p < 0.05$ (*)]. BL= baseline, TB= TB sonicate, MVS= *M. vaccae* sonicate, cont= unstimulated control wells and error bars represent range.

Table 7.1. IL-4 mRNA levels in PBMCs stimulated with *M. tuberculosis* sonicate.

Cytokine	Time-point	TB vs (negative control) or [MVS] median; range mRNA copy number	P value
IL-4 mRNA	D1/2 vs BL	460; 40-7673 vs (BL 12; 1-63)	P= 0.02
	D1/2	460; 40-7673 vs (5; 1-141)	P= 0.006
	D1/2	460; 40-7673 vs [19; 10-261]	P= 0.03
	D3/4	145; 1-4451 vs (1; 1-31)	P= 0.02
IL-4/IL-4 δ 2 ratio* (expressed as a log difference between cytokines)	D1/2	0.44 vs (-0.01)	NS
	D3/4	0.85 vs (-0.21)	NS
	D5/6	0.65 vs (0)	NS

*only median values shown for IL-4/IL-4 δ 2 ratio. MVS = *M. vaccae* sonicate. D = day. NS= non significant.

Table 7.2. Antigen-specific proliferative responses (overnight BrdU incorporation) in 8 patients with pulmonary tuberculosis.

Antigen	Median (range) Stimulation Index
TBS	1.3 (0.6-3.5)
MVS	1.1 (0.5-3.5)
PHA	6.2 (4-26)
Negative control	1.0 (1.0-1.0)

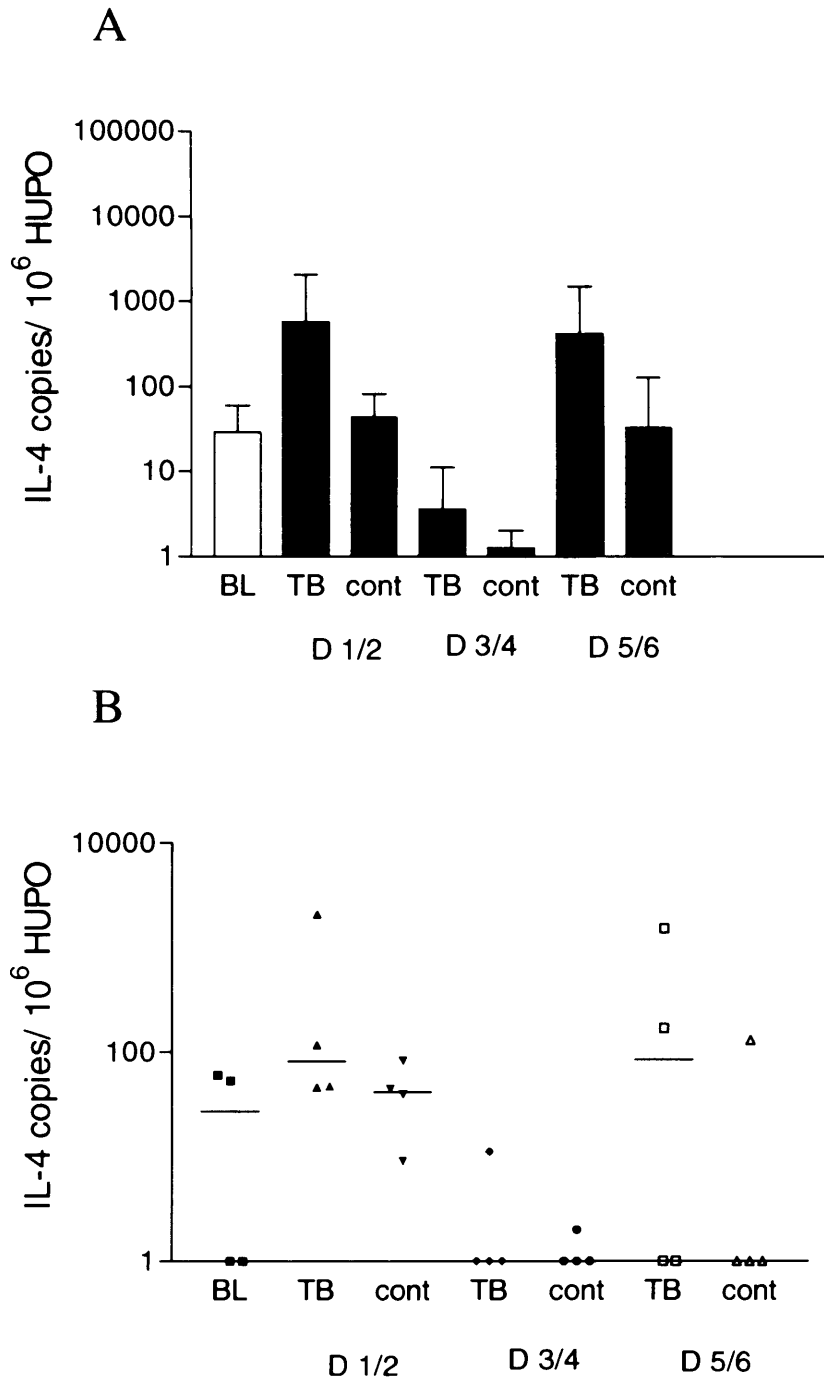


Figure 7.2. Cultures of blood mononuclear cells from 4 healthy control subjects (A shown as a bar graph and B shown as a scatter plot). IL-4 mRNA expression was higher in TB antigen stimulated wells than unstimulated wells (non-significant). BL= baseline, TB= TB sonicate, cont= unstimulated control wells, error bars represent range and horizontal lines represent medians.

7.3. Discussion

7.3.1. IL-4: exploring the cause effect relationship

Is the IL-4 response in TB an irrelevant consequence of infection and general immunological activation, or is it significantly involved in reducing protection and increasing immunopathology? Simple experiments where mice were pre-immunised, so that they had small Th2 responses to one or several components of *M. tuberculosis* before infection, have shown that pre-existing Th2 activity leads to increased severity of infection and to death [495, 496]. Wangoo *et al.* demonstrated that even a single epitope inducing a Th2 response can undermine the immune response [16]. In this transgenic mouse model polarised Th1, Th0 or Th2 cells, specific for a single 16 amino acid ovalbumin epitope, were transferred to recipient animals that were challenged with recombinant *M. tuberculosis* expressing the ovalbumin epitope, or with a control strain expressing an irrelevant epitope. Mice receiving Th1 cells before challenge were unaffected, while Th2 recipients showed enhanced weight loss and lung fibrosis and increased lung weight during acute high-dose infection with *M. tuberculosis*. Further studies in BALB/c IL-4 knockout mice proved that the absence of IL-4 led to diminished bacterial growth, and confirmed that IL-4 is directly involved in TNF- α -mediated immunopathology and fibrosis in this strain [14]. Interestingly, although other authors have not studied IL-4 knockout BALB/c mice, it was found that STAT6 (signal transducer and activator of transcription 6) knockout BALB/c mice respond normally to aerosol infection with *M. tuberculosis* [497]. This implies that the relevant functions of IL-4 (fibrosis and enhanced toxicity of TNF- α) might be signalled through 4PS/IRS-2 or IRS-1 rather than via STAT6 [498]. There is accumulating data that a DNA vaccine expressing hsp65 reduces pathology, enhances cure, shortens the duration of treatment and is effective against multidrug-resistant bacilli (MDR-TB) in infected mice [403, 499]. These beneficial effects are accompanied by the down regulation of IL-4 responses. Moreover, IL-4 neutralizing antibody showed enhanced bacterial elimination and therapeutic benefit in mice [500]. Collectively, these murine data suggest that the Th2 response in TB is unlikely to be an irrelevant bystander one.

This clear cause effect relationship in murine models has been more difficult to prove in humans. Collectively, published literature (reviewed in chapter 5) and the data presented in this thesis (chapter 5 and 6) demonstrate that both in BAL and peripheral whole blood, and their cell sub-populations, there is a mixed Th1/Th2 cytokine profile. However, this in itself does not imply a causal association between *M. tuberculosis* and IL-4. Demonstrating a relationship between IL-4 and the extent of disease (chapter 4 and 5) may also be insufficient (whether IL-4 is causal or an inflammation-related epiphenomenon, a greater degree of pathology would portend higher IL-4 levels). However, in addition to showing an association between IL-4 (whole blood and T cells) and radiological disease extent, demonstrating that TB antigen actually drives IL-4 in PBMCs from donors with active disease, supports the contention that IL-4 is not simply a bystander effect. Recently published human data showing that health care workers with pre-existing IL-4 responses have an increased rate of progression to active TB compared to those who do not [15] and that IL-4 gene polymorphisms may modulate disease severity and presentation [228], further supports this notion.

7.3.2. Sonicated TB antigen preferentially drives IL-4

Although data are limited, studies have shown that PBMCs or peripheral T cells stimulated with TB antigen (heat killed or sonicated or specific antigens or PPD), express IL-4 detected by RT-PCR [186], ELISPOT [18], or flow cytometry [21, 29, 347, 348]. Furthermore, hypervirulent Beijing *M. tuberculosis* strains cause human monocytes to express IL-4 [290]. By contrast, 3 earlier studies (before 1998) using RT-PCR failed to find IL-4 in TB antigen stimulated PBMCs or peripheral T cells [28, 30, 355]. The technical and other reasons for the failure to find IL-4 in these studies [28, 30, 355] have already been discussed in chapter 5, section 5.4.1). Collectively, however, the data suggest that more sensitive methods such as RT-PCR and ELISPOT are required for the detection of IL-4 in subjects from developed countries [18, 186] compared to FACS in subjects from developing countries [21, 29, 347, 348] (already discussed in chapter 1, section 1.7.5.2). The findings presented in this chapter confirm the findings of previous investigators that TB antigen can drive IL-4 in subjects with active disease.

However, the expression of IL-4 δ 2 has not previously been investigated. As already mentioned in chapter 1, 5 and 6, the expression of IL-4 δ 2 mRNA is increased in unstimulated PBMCs from healthy donors who have LTBI, but not household contacts or those without LTBI, in The Gambia and Ethiopia [360, 361]. This suggests the possibility that blocking the effects of IL-4 through increased production of IL-4 δ 2 might have a protective function. The observation that TB antigen preferentially drives IL-4 rather than the antagonist IL-4 δ 2 may fit with this hypothesis. However, further experiments are required to confirm or refute this contention. The toxicity of TNF- α in an IL-4 knockout mouse infected with *M. tuberculosis* has been shown by the addition of recombinant IL-4 [14]. Similar experiments conducted with IL-4 δ 2 and follow-up of the Gambian and Ethiopian cohort for disease progression would help to clarify the role of IL-4 δ 2 in tuberculosis. Murine experiments are already underway in Mexico to answer some of these questions and preliminary data indicate that some of the previously found 'IL-4' is, in fact, IL-4 δ 2 (Rogelio Hernandez Pando, personal communication).

7.3.3. Are there specific antigens that preferentially drive IL-4 or IL-4 δ 2?

Having demonstrated that TB antigen, and not an environmental mycobacterium, drives IL-4 the next step will be to determine which specific antigenic components do this. Such antigens can be excluded from vaccine preparations (e.g. recombinant BCG), or, administered together with adjuvants that can drive Th2 suppressing regulatory cells [257, 402] or induce a Th1 response rather than one driving IL-4. Alternatively, an intriguing possibility is to find adjuvants or antigens that drive IL-4 δ 2, thereby tipping the balance in favour of the IL-4 antagonist. Putative strategies to identify such antigens (IL-4 and IL-4 δ 2-specific) include the screening of TB antigen-specific IgE responses or *E. coli* DNA libraries expressing *M. tuberculosis* antigens. It has been shown that certain antigens can drive IL-4 and function as promoters of Th2-cell responses. The 16kDa heat-shock protein (α -crystallin) of *M. tuberculosis* contains epitopes that are preferentially recognised by Th2 cells from patients with TB [501]. In some individuals the 30kDa and 19kDa may induce IL-4 [18] and whilst IL-4 responses to antigen 85B may be detected 2 months after anti-TB treatment but not at diagnosis [502]. Consequently, if the hypothesis that IL-4 subverts protective

host responses is correct, vaccines that boost antigen 85 responses are likely to be ineffective in their current format.

Most known vaccine candidates that are effective as prophylactic vaccines in mice, including BCG and preparations of *M. tuberculosis* itself, are ineffective as therapeutic vaccines [148, 149]. The history of this observation goes back to Robert Koch, who observed necrosis (the Koch Phenomenon) in guinea-pigs and humans given injections of 'Old Tuberculin' (reviewed in [503]). However, there are two preparations which downregulate IL-4, that are active therapeutically in murine TB. One, discussed in section 7.3.1 above, is a DNA vaccine based on heat-shock protein 65 (hsp65) of *Mycobacterium leprae*, which reduces bacterial numbers in chronically infected mice and concomitantly reduces IL-4 ELISPOT+ T-cell numbers [499]. The other exception, heat killed *Mycobacterium vaccae*, markedly reduces IL-4 expression when administered after intrapulmonary infection [365]. Indeed, in the current study TB sonicate drove significantly greater IL-4 mRNA expression compared with *M. vaccae*, whose major mode of action is suppression of Th2 responses by driving CD4+CD45RB^{low} regulatory T cells [402]. Multiple doses of *M. vaccae*, in contrast to a single dose that does not enhance sputum conversion rates [504, 505], are being used therapeutically by the Chinese on a large scale. In a large RCT *M. vaccae* enabled successful shortening of the standard anti-TB regime to 4 months [506] and improved 6 month culture conversion rates in MDR-TB [507]. A multiple dose *M. vaccae* trial in HIV+ subjects is currently in progress in Tanzania.

7.3.4. Choice of experimental protocol

Initial experiments suggested that 24 to 48 hours was the peak time-point at which IL-4 responses could be elicited in antigen driven PBMC cultures. Nevertheless, a seven day culture period was chosen to evaluate proliferative responses and protein measurements by ELISA, which may lag behind mRNA expression. The optimal antigen dose was selected by measuring cell proliferation, assessing cell viability with trypan blue and microscopic inspection of cultures (a final concentration of TB sonicate exceeding 50 µg/ml was found to be toxic).

We viewed exposed but not latently infected controls as a group 'resistant' to TB

infection, and considered this group as an appropriate control group relative to 'susceptible' subjects that developed progressive disease. Given the raised IL-4/IL-4 ratio recently described in latently infected contacts [360, 361], it would have been desirable to simultaneously study a control group with LTBI in addition to the chosen one (exposed but not latently infected). However, resource constraints and related studies in Africa (VACSIS/ VACSEL) precluded the inclusion of this group. Further limitations of the antigen driven culture study include the small number of patients (8 TB patients and 4 controls) and groups that were incompletely matched with respect to ethnicity. However, the addition of each patient (with the relevant control) would have added 144 additional samples (3 wells in triplicate, for 4 timepoints, in 2 donors for 3 genes) for processing (RT-PCR and ELISA). This was not logistically possible given the resources committed to this study.

7.3.5. Detection of IL-4 and IFN- γ by RT-PCR vs ELISA

IL-4 was largely undetectable by ELISA in culture supernatants. This was not surprising given the poor sensitivity of ELISA for IL-4, which is undetectable even when blocking antibodies are used [34] because of *in vitro* consumption by widely distributed cellular (and soluble) receptors [415]. This failure to detect IL-4 in culture supernatants, whilst confirming expression by RT-PCR in antigen driven PBMCs, has been confirmed by other investigators [26, 186]. In TB patients antigen driven IFN- γ mRNA expression was not significantly different compared to control subjects (similar to findings in whole blood outlined in chapter 5) and TB-specific proliferative responses were unremarkable. Poor antigen-driven proliferative and IFN- γ responses in peripheral blood or PBMCs of donors with TB has been well documented by other investigators [28-30, 78]. This is likely to be due to translocation of antigen-specific IFN- γ producing cells to the lungs where there are high IFN- γ levels (demonstrated in chapter 5) and strong proliferative responses [264]. Though not possible due to logistical and budgetary constraints, it would have been enlightening to compare antigen driven responses in PBMCs and alveolar lavage cells where IL-4 mRNA levels are increased by almost 2 logs (chapter 5). Cytokine production may also be TB strain-dependent with some strains driving little or no IFN- γ production compared to others, even in healthy volunteers [508].

7.4. Conclusion

The data presented in this chapter indicate that, in patients with active disease, TB antigen, preferentially drives IL-4 rather than the antagonist IL-4 δ 2. This together with the association between IL-4 mRNA levels and radiological disease extent (chapter 5 and 6) supports the contention that IL-4 is not simply a bystander effect. These data, together with those latently infected contacts that remain healthy [360, 361], support the hypothesis that IL-4 δ 2 expression may determine the balance between infection and disease. The implications for vaccine design will be to screen for antigenic components (that drive IL-4, or perhaps IL-4 δ 2), which can then be manipulated to downregulate the subversive Th2 component but at the same time augmenting the Th1 response.

CHAPTER 8: IL-4, IL-4 δ 2 AND IFN- γ PROFILES IN PATIENTS CO-INFECTED WITH PULMONARY TUBERCULOSIS AND HIV

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8.1. Introduction

Tuberculosis and HIV are closely interactive pandemics that continue to flourish despite the availability of effective chemotherapy. Accordingly, immunotherapy in the form of widely available vaccines or immune modifiers would be crucial adjuncts for disease containment [509]. However, developing a successful HIV or TB vaccine has been more challenging than expected. The mechanisms underlying HIV related CD4+ T cell dysfunction and progression to AIDS remain unclear. Furthermore, the mechanisms governing disease progression and correlates of protective immunity are poorly understood.

It has been suggested that a subversive Th2 component, which undermines the dominant Th1 response, is associated with the pathogenesis of TB [236], HIV [510, 511] and progression to AIDS [512]. However, the presence and significance of Th2 cytokines in HIV is controversial [513]; moreover, Th2 related data in HIV [510, 514-517] and HIV-TB co-infection [350, 353, 408, 518] is sparse and discordant. More importantly, the available literature fails to take into account the presence of IL-4 δ 2, which confounds the interpretation of existing Th2 data. However, IL-4 δ 2 expression has not previously been studied in HIV or HIV-TB co-infection. Understanding the

relevant contribution of IL-4/IL-4δ2 in TB and HIV may be important for intervention strategies, which modulate the Th1/Th2 balance in both diseases.

The expression of IL-4δ2 in whole blood and lung lavage, of donors with TB-HIV co-infection, HIV alone and non-HIV infected healthy volunteers, is outlined in this chapter.

8.2. Methods

8.2.1. Patients and samples

Twenty patients with clinical and radiological features of pulmonary TB (sputum or lavage fluid positive by nucleic amplification test or culture for *M. tuberculosis* [6]) and HIV co-infection, 13 of whom underwent bronchoscopy and bronchoalveolar lavage (BAL), were recruited. Control donors were 20 otherwise healthy HIV-positive volunteers who were matched to the index cases for age (within 4 years), sex, and CD4 count (<200, 200 to 350 or > 350 cells/ml); and 20 HIV-negative healthy volunteers. Six volunteers from each control group (HIV positive and negative) underwent BAL. Thus, whole blood was obtained from 20 HIV-TB co-infected subjects, 20 HIV+ volunteers without opportunistic infection (OI) and 20 healthy controls; BAL was obtained from 13 HIV-TB co-infected patients, 6 HIV+ volunteers and 6 HIV negative healthy controls.

In all HIV+ donors other concomitant viral (hepatitis, CMV, EBV), bacterial (syphilis, bacterial culture of BAL fluid in appropriate donors), fungal (cryptococcosus, histoplasmosis) and parasitic infections (toxoplasmosis and screen for stool parasites if indicated) were excluded by PCR, serology, cytology and culture of relevant biological samples (blood or BAL). Plasma HIV viral load (LCx HIV RNA Quantitative assay; Abbott Laboratories, North Chicago, USA), total peripheral blood and BAL CD3+, CD4+ and CD8+ counts (TruCOUNT™, BD Biosciences, San Jose, CA) were performed in all HIV positive participants (performed by technicians in the Dept. Virology, Royal Free Hospital). Whole blood, obtained from all participants before or within 10 days of initiating anti-TB treatment and also from 10

TB-HIV co-infected patients after anti-TB treatment, was immediately transferred to Paxgene tubes (Qiagen, Germany) to fix the RNA profile. BAL and radiographic scoring was performed as outlined in the general methods (chapter 3).

8.2.2. ELISPOT, ELISA and RT-PCR

Latent TB infection was excluded in HIV+ and HIV- control subjects by overnight T-cell IFN- γ responses to TB-specific antigens (T-SPOT TB, Oxford Immunotec, England). In addition ELISA for sCD30, IL-4 and IFN- γ was performed on ~10 fold concentrated lavage supernatants as described in chapter 3. RNA extraction, reverse transcription and real-time quantitative RT-PCR for IL-4, IL-4 δ 2 and IFN- γ were performed on all blood and BAL samples as previously described after quantification and quality control of RNA templates (chapter 3). Data was analysed according to the methods described in chapter 3.

8.3. Results

8.3.1. Demographic and laboratory characteristics

The baseline demographic and laboratory characteristics were not significantly different in the 3 patient groups (table 8.1). However HIV-TB co-infected patients had higher viral load (median copies/ml plasma) than HIV+ controls (77760 vs 50, $p=0.0001$).

Table 8.1. Demographic and laboratory characteristics of study participants. Figures accompanied by parentheses are means (SD).

Characteristic	TB+HIV+	HIV+	HIV -
Age (years)	37.1 (8.6)	36.2 (5.7)	34.9 (14.7)
CD4 count (cells/mm ³)	223 (201)	279 (198)	n/a
% male	50	42	57
% Black African	79	74	50
% using HAART	15	40	n/a

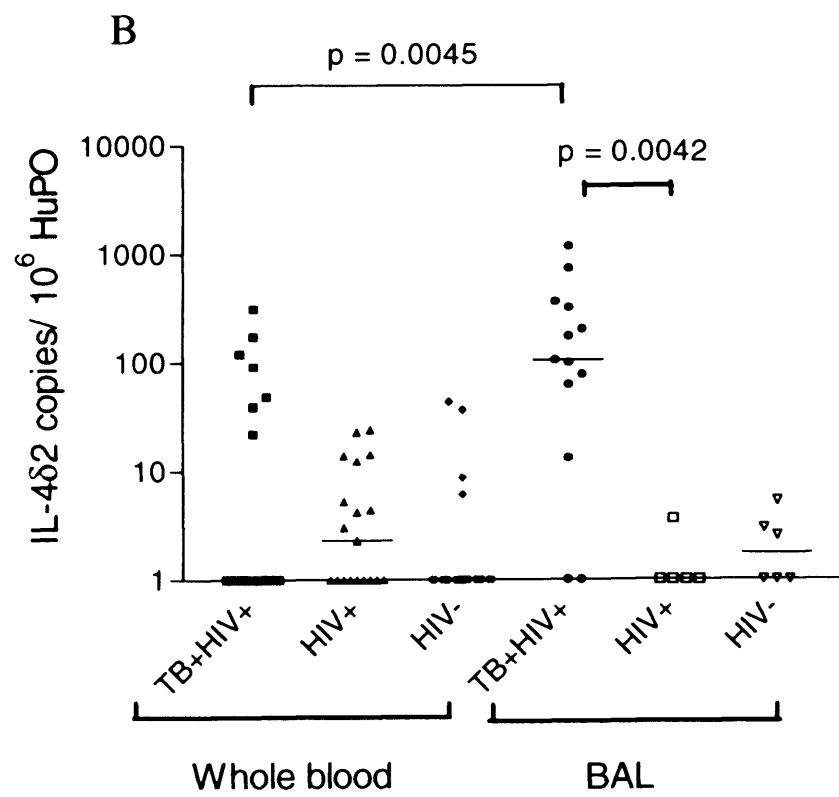
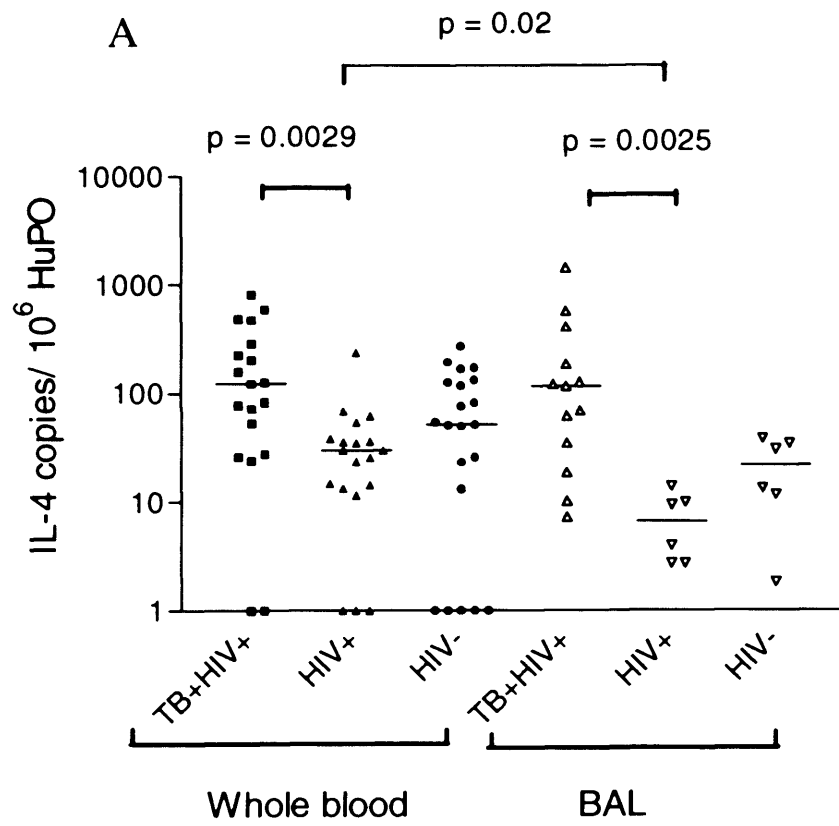
8.3.2. HIV positive and HIV negative healthy controls

In HIV+ controls without OI vs non-HIV controls IL-4, IL-4 δ 2 and IFN- γ were similar in whole blood and lung lavage fluid (figure 8.1 A, B and C), even when paired samples from both compartments were compared. mRNA levels did not differ significantly in those taking HAART compared to those who were not.

8.3.3. HIV-TB co-infected patients and HIV positive controls

By contrast mRNAs for IL-4 and IFN- γ were increased in both compartments of TB-HIV co-infected donors, compared to the HIV+ controls [$p < 0.003$ for IL-4 (~5 fold increase in blood and ~17 fold increase in lavage) and $p < 0.02$ for IFN- γ (>350 fold increase for blood and >1300 fold increase for lavage); figures 8.1 A and C)]. However, IL-4 δ 2 mRNA levels were similar in the blood whether the HIV+ individuals were TB co-infected or not, whereas levels were much higher in lung lavage from co-infected donors ($p < 0.005$, ~100 fold increase, figure 8.1 B). Consequently, in co-infected patients the IL-4/ IL-4 δ 2 ratio was significantly higher in whole blood than in lung lavage ($p = 0.0006$), where the dominant form of “IL-4” was in fact the antagonist, IL-4 δ 2 (figure 8.2).

sCD30 and IL-4 were undetectable by ELISA in BAL from most HIV+ donors, whether TB-coinfected or not. By ELISA, IFN- γ was increased in BAL from some co-infected patients, but undetectable in 50%. Thus, ELISA results were unhelpful.



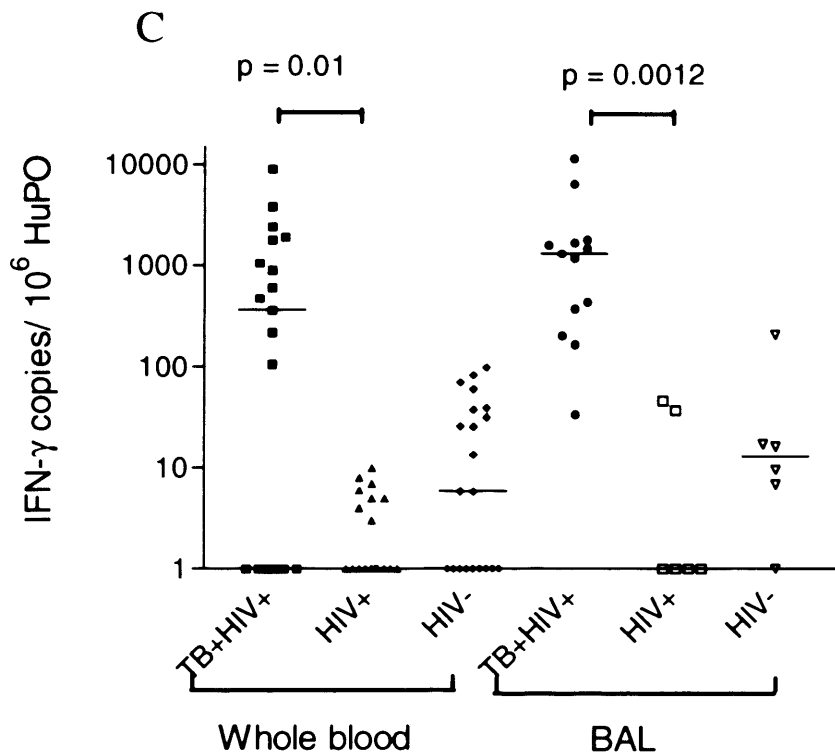


Figure 8.1. A, B and C. IL-4 (A), IL-4 δ 2 (B) and IFN- γ (C) mRNA levels (expressed per million copies of mRNA encoding Human acidic ribosomal protein) in whole blood and alveolar lavage at the start of anti-TB treatment. Expression of IL-4 and IFN- γ were significantly increased in patients with tuberculosis compared to controls. IL-4 δ 2 was elevated in the lung lavage but not in whole blood of co-infected patients compared to controls.

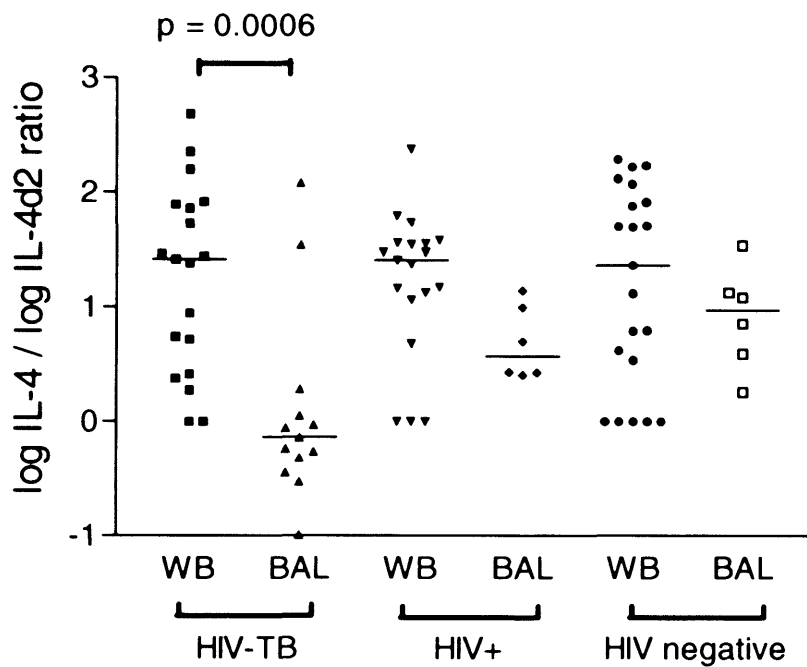


Figure 8.2. IL-4/IL-4 δ 2 ratio (expressed as a log difference between cytokines) in whole blood (WB) and alveolar lavage cells (BAL). The IL-4/IL-4 δ 2 ratio was significantly higher in BAL compared to whole blood in subjects with TB-HIV infection.

8.3.4. Relationship to clinical, laboratory and treatment parameters

In HIV-TB co-infected patients the IL-4/IFN- γ mRNA ratio was similar in whole blood and lung lavage and the latter correlated with pre-treatment radiological disease extent ($p = 0.02$, figure 8.3). It was not feasible to evaluate the relationship between cytokine mRNA levels and cavitary disease as there were only 2 patients with cavitation discernable on chest x-ray.

IL-4 and IL-4 δ 2 mRNA levels were independent of blood and lavage CD4 and CD8 counts, blood viral load, the presence of disseminated tuberculosis and use of HAART. There was no relationship between the CD4 count and immunological parameters (IL-4, IL-4 δ 2 and IFN- γ mRNA levels, and the Th2/Th1 ratio were not significantly different in subjects with CD4 counts < 200 vs > 200).

Following short course anti-TB treatment IL-4 and IFN- γ mRNA levels did not significantly change whilst IL-4 δ 2 levels significantly increased ($p=0.01$, ~85 fold increase, figure 8.4 A-C).

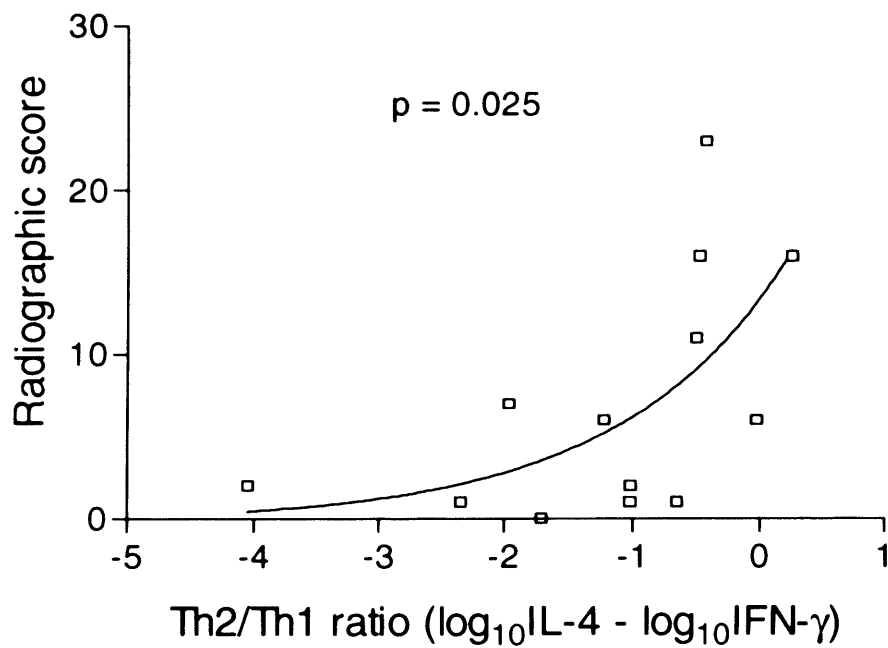
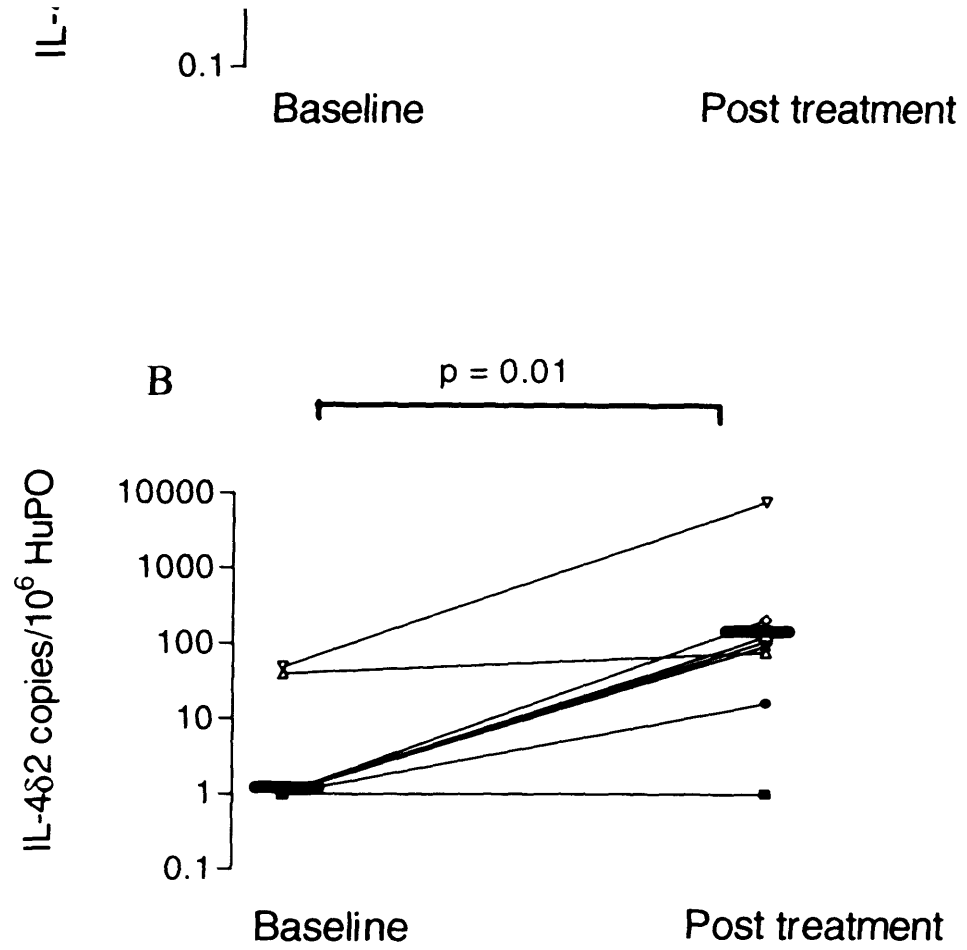


Figure 8.3. Correlation between radiological disease extent and the Th2/Th1 (IL-4/IFN- γ) ratio in BAL.



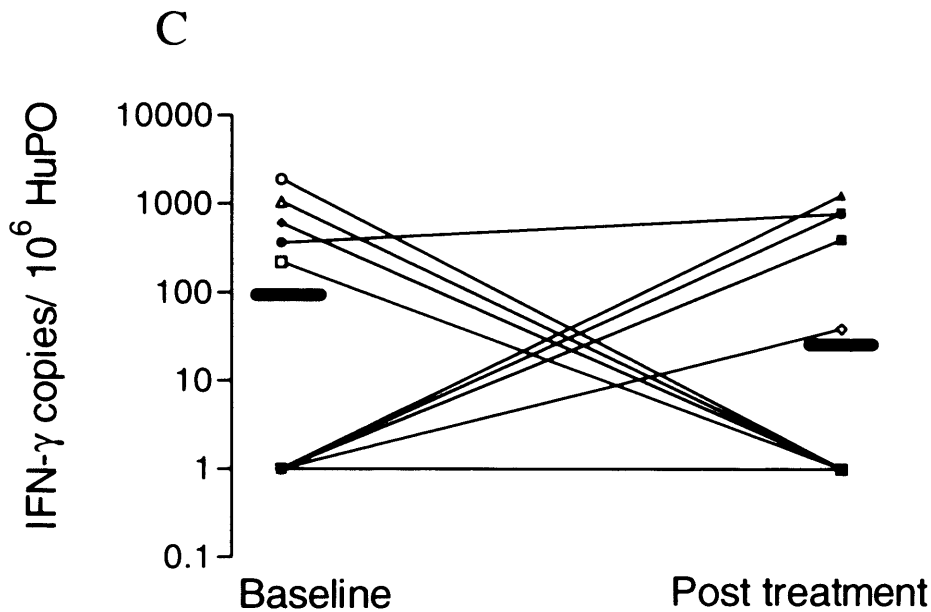


Figure 8.4 A-C. Comparison between IL-4 (A), IL-4 δ 2 (B) and IFN- γ (C) mRNA levels at baseline and after anti-TB treatment (n=10). At ~ 6 months post treatment IL-4 and IFN- γ mRNA levels were non-significantly changed from baseline but IL-4 δ 2 levels significantly increased (p=0.01, ~85 fold increase).

8.4. Discussion

8.4.1. IL-4 in asymptomatic HIV infection

The 'Th1 to Th2 switch' hypothesis proposes that immune dysregulation facilitating infection and progression to AIDS is dependent on the balance between Th1 and Th2 responses to HIV [512]. There is also evidence that Th2 cells are more permissive to HIV infection [519]. However, published studies regarding IL-4 are conflicting with some studies supporting [510, 515, 516, 520] and some opposing [514, 517] the 'Th1 to Th2 shift' hypothesis. In asymptomatic HIV+ subjects there was a progressive decline in IL-2 and IFN- γ responses and increased IL-4 responses, detected by conventional RT-PCR [510] or flow cytometry [515, 520], in mitogen stimulated PBMCs. Similar findings were noted in primary HIV infection but not in chronic asymptomatic infection [516]. By contrast, IL-4 was largely undetectable by RT-PCR in unstimulated PBMCs in primary HIV infection [514], or by flow cytometry in

chronic asymptomatic HIV infection, even at CD4 counts less than 200 cells/mm³ [517]. Serum IL-4 levels were not elevated in the serum of patients with chronic asymptomatic infection [521]. Consequently, the issue of whether a switch to Th2 responses facilitates the progression of HIV remained controversial.

Why are the data discordant? Collectively, these studies did not distinguish between the agonist, IL-4, and antagonist, IL-4δ2. Moreover, stimulation protocols, which are Th1 biased may have down regulated IL-4 responses in *in vitro* cultures. Interpretation is further complicated by heterogeneity in the stage of HIV studied (primary infection, stable HIV or AIDS), the site of sample acquisition (peripheral blood vs disease site), geographical location (Europe and USA vs Africa) and the type of assay used to measure IL-4 (conventional RT-PCR, ELISA, flow cytometry). The drawbacks of immunoassays and unreliability of conventional RT-PCR protocols have been summarised (chapter 2, table 2.2). In the current study mRNA levels of IL-4 and IL-4δ2 were measured in *unstimulated* cells from both blood and lung lavage, using a validated probe based real-time quantitative assay (chapter 2), in asymptomatic HIV+ subjects over a wide range of CD4 counts. The Th2 profiles were similar in HIV infected and non-infected healthy volunteers and this was independent of disease stage (CD4 count), viral load or use of HAART. The data presented here argue against the implication of Th2 cytokines in the pathogenesis of HIV in the absence of other opportunistic infections. This implies that immunotherapeutic interventions based on the Th1/Th2 shift are unlikely to be useful in asymptomatic HIV+ subjects. Nevertheless, it remains possible that cells from HIV-infected donors release more IL-4 (or IL-4δ2; the assays used would have measured both together) when stimulated *in vitro* with mitogens [515, 516]. Moreover, whether the findings presented here apply to primary HIV infection remain unclear.

8.4.2. IL-4 expression in HIV-TB co-infected subjects

Considering that TB is the commonest OI in HIV and that in some parts of the world more than 60% of TB patients are co-infected with HIV, it is remarkable that there are few published data about Th1/ Th2 cytokine profiles in co-infected patients. Analogous to asymptomatic and primary HIV infection the data are conflicting. Two studies reported a shift to a type 2 cytokine profile [518, 522] whilst three others

found reduced type 1 responses but no enhancement in IL-4 responses [353, 408, 523]. These studies used ELISA to detect IL-4 in *in vitro* cultures and conventional RT-PCR protocols; the same drawbacks discussed in the previous section are applicable here. The current study found that HIV-TB co-infected patients, but not HIV+ controls, had significantly higher levels of IL-4 mRNA in blood and alveolar lavage cells. That these observations are biologically meaningful is corroborated by the correlation of the lung Th1/ Th2 (IL-4/ IFN- γ) ratio with pre-treatment radiological scores. Nevertheless, the association of IL-4 mRNA levels with disease extent does not necessarily imply a cause-effect relationship and further studies will be required to clarify this issue. Whether these observations apply only to TB or also to other opportunistic infections remain unclear. Published reports investigating Th1/Th2 cytokine profiles in other OI such as PCP, cryptococcosis and toxoplasmosis are too sparse to draw any meaningful conclusions.

The mechanisms that underpin increased viral replication during TB co-infection are poorly understood. The data presented here confirm that in HIV+ hosts, TB induces IL-4 as it does in HIV sero-negative TB (chapter 5), and the profile of IL-4 and IL-4 δ 2 in HIV-TB co-infection is similar to that found in sero-negative TB (chapter 5). IL-4 may facilitate HIV progression by up-regulating HIV co-receptors, encouraging syncytium-inducing strains [524], inhibiting anti-viral α -defensins [525, 526] and activating viral transcription [524, 527]. Furthermore, in HIV-TB, as in any co-infection, there is enhanced viral cellular entry due to cellular activation and increased viral transcription mediated by transcription factors like NF- κ B [513]. The high IFN- γ levels found in co-infected patients may also facilitate HIV progression by up regulating HIV co-receptors [528] though there was no correlation between plasma viral load and IFN- γ mRNA levels. However, plasma viral load is only a surrogate marker of total viral burden and viral load was not measured at the main site of disease, the lung.

Not surprisingly, ELISA for IL-4 was unhelpful in the current study. Immunoassays have poor sensitivity in detecting protein in BAL due to the considerable dilutional effect in lavage fluid (this point has been discussed in chapter 5, section 5.4.2).

8.4.3. IL-4 δ 2 mRNA expression

In contrast, to IL-4 mRNA expression, IL-4 δ 2, the antagonist, was increased predominantly in the lungs but not in the blood. Consequently, co-infected patients had a significantly higher IL-4/ IL-4 δ 2 ratio in whole blood compared to lung lavage. As IL-4 enhances immunopathology in animal models [14, 16, 364] and human disease [19, 356], it is not unreasonable to speculate that the paucity of immunopathology (necrosis and cavitation) seen in HIV may be relate to the high IL-4 δ 2 levels in the lungs. However, it is recognised that the genesis of immunopathology is complex and may be modulated by different cell types (T cells and granulocytes), cytokines (IL-4, TNF and TGF- β), protease dysregulation and other factors (toxicity of mycobacteria, Koch phenomenon and Schwartzman reaction) (reviewed in chapter 1). Nevertheless the relationship between IL-4 δ 2 and immunopathology in HIV merits further investigation. In the current study it was not feasible to determine the relationship between IL-4 δ 2 and cavitation as there were only 2 individuals in whom cavitary lesions were visible on CXR. However, it is possible that a larger cohort and use of more sensitive methods such as CT scans may have detected an association. BAL samples from the current study have been banked for future experiments, which will seek to determine the relationship between Th2 cytokines and markers of lung remodelling (TGF- β , MMPs, procollagen peptide III).

8.4.4. Response to anti-TB treatment

Analogous to the changes seen in HIV negative subjects (chapter 5), completion of chemotherapy was accompanied by a significant increase in IL-4 δ 2 mRNA levels and a reduced IL-4/ IL-4 δ 2 ratio. Further studies are warranted to evaluate the utility of this ratio as a marker of disease activity or successful treatment. If this is borne out it may be of value for future trials of new therapeutic interventions. The observation together with those in latently infected contacts that remain healthy [360, 361], raises questions about whether IL-4/IL- δ 2 ratio could be used as a correlate of protective immunity. This point has been discussed in relation to HIV negative subjects in chapter 5.

8.4.5. IFN- γ levels in blood and BAL

The notion that there are sufficient Th1 responses in TB and further enhancement is unlikely to increase protection, was discussed in detail in chapter 5. In HIV-TB co-infection, compared to HIV+ controls, IFN- γ mRNA levels were significantly higher in peripheral whole blood and the lungs. Furthermore, co-infected subjects with CD4 counts < 200 cells/mm³ did not have significantly lower IFN- γ mRNA levels than those having counts > 200 cells/mm³. Antigen specific IFN- γ responses are also robust in the lungs, even at low CD4 counts (RAM Breen, personal communication). In contrast to the data presented here, the only other study to evaluate expression in BAL fluid [468] found that IFN- γ mRNA levels were reduced in 4 co-infected subjects compared to HIV negative controls. However, a semi-quantitative protocol and the small number of patients were limitations of this study. Collectively, the findings presented here and in chapter 5 suggest that Th1 cytokines like IFN- γ are unlikely to be the limiting factor in protective immunity in both HIV negative and HIV+ subjects with tuberculosis.

8.5. Conclusion

The data presented in this chapter suggest a role for Th2 cytokines in the pathogenesis of HIV-TB co-infection but not in otherwise stable HIV infection. Further studies are required to determine if this applies to other HIV related opportunistic infections, and whether the selective increase in IL-4 expression in the periphery of co-infected patients contributes to increased HIV replication. The relationship between the IL-4/IL-4 δ 2 ratio and immunopathology, disease activity and protective immunity warrants further investigation. Collectively, the findings presented here have implications for the design of immunotherapeutic interventions that seek to slow disease progression by modulating the Th1/Th2 balance in HIV and HIV-TB co-infection.

CHAPTER 9: GENERAL DISCUSSION

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9.1. Introduction

The studies related to pulmonary tuberculosis, performed in fulfilment of this thesis, aimed to further the understanding of those Th1 and Th2-specific responses and regulatory pathways, about which little or no previous human data existed.

9.1. Discordant IL-4 data in relation to TB

There are many technical difficulties when measuring and interpreting data in relation to low copy number cytokines like human IL-4 (summarised in table 2.1). Furthermore, published literature is characterised by unreliable mRNA quantification using conventional RT-PCR protocols (drawbacks are listed in table 2.2), variability in sample acquisition (periphery rather than the disease site) and geographical location (implications discussed in chapter 1, section 1.7.5.2), and a general failure to distinguish between IL-4 and IL-4 δ 2. Accordingly, it is not surprising that IL-4-related data are discordant. The first step in addressing this problem was to design an accurate and reproducible assay that would circumvent these problems (chapter 2 and 3). Real-time RT-PCR was the obvious choice as it addressed most of these drawbacks and could simultaneously distinguish between IL-4 and IL-4 δ 2, for which there are no available discriminatory immunoassays. As IL-4 is active at concentrations \sim 300 fold below that of IFN- γ [413], the technique had to be precise enough to detect even small inter-group differences yet concurrently not create artefactual ones (a possible effect of data normalisation when using PCR). However, as there was no previously established reference for standardising data generated from tuberculosis samples, it was first necessary to validate a suitable reference gene

(chapter 2, part 1). It was also demonstrated, for the first time, that use of an unselected conventional reference (GAPDH) could obscure significant inter-group differences or artefactually generate them (chapter 2, part 2) when measuring IL-4 mRNA levels. To generate interpretable data, in the current study, samples were simultaneously acquired from peripheral blood *and* the lung. Furthermore, *unstimulated* cells were immediately ‘fixed’ to ensure a reliable reflection of *in vivo* expression profiles (chapter 3). A number of other technical measures were taken (table 3.1), some often ignored in published studies, to ensure the generation of accurate, reproducible and biologically meaningful data. Using this carefully validated protocol it was unequivocally shown that subjects with pulmonary tuberculosis, in the periphery and in the lung, have a mixed Th1/ Th2 profile compared to healthy volunteers. The methods developed here, including the use of a validated reference, could be applied to study other biological markers (e.g. transcription factors, signalling molecules and cytokines) in tuberculosis. It would also be useful for study of BAL where dilution of cytokines hampers their detection (discussed in detail in chapter 5, section 5.4.2).

9.2. What is the significance of IL-4 seen in TB?

The difficulty with immunological studies in TB, and this also applies to other inflammatory conditions, is to determine whether a biological marker of interest is causative or an epiphenomenon (bystander effect due to widespread pathology or inflammation). The same caveat applies when demonstrating an association between IL-4 and disease extent, as shown in the current study. Furthermore, murine data which show a direct cause-effect relationship for IL-4 (discussed in detail in chapter 7, section 7.3.1), may not be entirely relevant to human tuberculosis. *In vitro* studies using human cells often have discordant findings because of variable culture conditions and antigen preparations (reviewed in chapter 5). Finally, demonstrating a strong cause-effect association in human studies does not necessarily ensure therapeutic utility. For example human anti-IL-5 antibody, initially validated in knock-out mice, effectively abrogated human airway eosinophilia but showed little clinical benefit in trials for bronchial asthma [529]. Collectively, these precepts indicate that proving a direct cause-effect relationship between IL-4 and human TB can be tricky.

Nevertheless, a recent study showed that individuals with pre-existing TB antigen-specific IL-4 responses had a high rate of progression to active TB [15]. In the current study, the association of the Th2 / Th1 ratio and sCD30 with radiological disease extent (chapter 5) supports the biological relevance of IL-4 mRNA levels. The failure of IL-4 mRNA to decrease in parallel with clinical and immuno-pathological improvement (radiology and concurrent increase in IFN- γ levels) argues against a bystander effect (chapter 5). Furthermore, the predominant T cell expression of IL-4 (chapter 6) and the induction of IL-4 by TB antigen (chapter 7) suggest a true antigen driven response. Collectively, these data support, but does not prove, the hypothesis that TB drives IL-4, within the context of a dominant Th1 response, to subvert protective host immunity. The hypothesis is summarised in figure 9.1 and if correct may help clarify a number of paradoxes and hitherto potentially unexplained phenomena in TB (summarised in table 9.1). Further studies are required to assess the contribution of regulatory responses (Th1 or Th2) in determining T cell profiles in active and latent TB (discussed in chapter 1, section 1.5). Moreover, studies are required to clarify the role of soluble IL-4R and natural antibodies to IL-4 in tuberculosis. It is possible that sIL-4R may represent a major pathway for regulating IL-4 protein levels in TB and sIL-4R may considerably prolong the half-life of IL-4 protein. This may partly explain the high levels of TB-related IL-4 found in developing countries. The same considerations apply to natural antibodies to IL-4 (sIL-4R and anti-IL-4 antibodies are discussed in section 1.6.4.2 and 1.6.4.3).

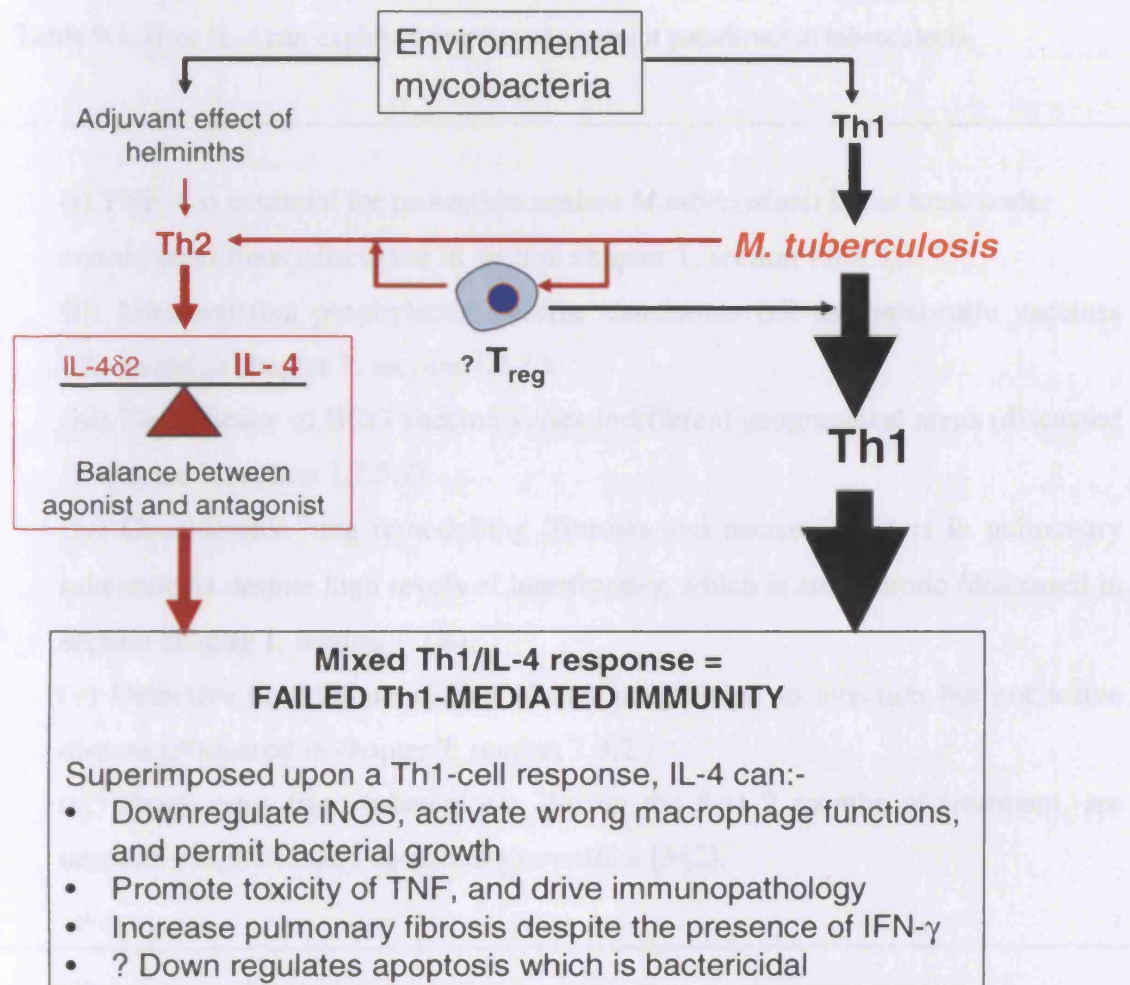


Figure 9.1. Tuberculosis and the interleukin-4 (IL-4) hypothesis. Environmental mycobacteria prime Th1 and Th2-cell responses, the latter being enhanced by helminth infections. Dose dependent exposure to *M. tuberculosis* will enhance both Th1 and Th2 cell responses. IL-4 may be partially counteracted by increased expression of the splice variant IL-4δ2, which occurs in latently infected individuals who do not develop disease. However, in others a mixed Th1-cell and IL-4 response might compromise cell-mediated immunity against *M. tuberculosis*. The result is a failure of protection and the development of disease with unusually high levels of IL-4 in developing countries. To what extent *M. tuberculosis* drives regulatory cells (Treg) to enhance Th2 responses remain unclear. An effective vaccine for citizens in developing countries might need to block the IL-4 response, rather than induce a Th1-cell response that is already present.

Table 9.1. How IL-4 can explain a number of apparent paradoxes in tuberculosis.

- (i) TNF- α is essential for protection against *M.tuberculosis* but is toxic under certain conditions (discussed in section chapter 1, section 1.7.4.1.).
- (ii) Most existing prophylactic vaccine candidates fail as therapeutic vaccines (discussed in chapter 7, section 7.3.3.).
- (iii) The efficacy of BCG vaccine varies indifferent geographical areas (discussed in chapter 1, section 1.7.5.2).
- (iv) Considerable lung remodelling (fibrosis and necrosis) occurs in pulmonary tuberculosis despite high levels of interferon- γ , which is anti-fibrotic (discussed in section chapter 1, section 1.7.4).
- (v) Defective host immunity that allows progression to infection but not active disease (discussed in chapter 7, section 7.3.2.)
- (vi) Death rates from tuberculosis, during the first 2 months of treatment, are unusually high in many developing countries [342].

9.3. Can the balance between IL-4 and IL-4 δ 2 determine disease outcomes?

As IL-4 is implicated in TB-related immunopathology and disease progression in mice [14, 16, 364, 368] and humans [19, 356], and IL-4 δ 2 is an *in vitro* antagonist of IL-4 [314], it is logical to ask whether the balance between the two (IL-4 and IL-4 δ 2) determines disease outcomes.

Proving this hypothesis in humans is challenging. The first clue came from the observation that subjects with LTBI who remain healthy express high levels of IL-4 δ 2 mRNA [360, 361] and fuelled speculation that the 'IL-4-IL-4 δ 2' hypothesis was tenable. Three observations in the current study support this contention. Firstly, the stability of IL-4, but not IL-4 δ 2 mRNA, is enhanced in peripheral blood (chapter 4).

In pulmonary tuberculosis this, previously undescribed, regulatory mechanism is likely to enhance the immunopathologic effect of IL-4. Secondly, TB antigen preferentially drives IL-4 rather than IL-4δ2 in cultures of peripheral mononuclear cells obtained from TB patients (chapter 5). Thirdly, unlike IL-4, IL-4δ2 increases significantly with treatment and clinical improvement, in both HIV negative (chapter 5) and HIV+ (chapter 8) subjects with tuberculosis. Moreover, HIV+ subjects, most of whom have no radiologically apparent lung cavitation, have high levels of IL-4δ2 in the lungs but not the blood (chapter 8). However, at apparent odds with this contention is the finding that the IL-4/ IL-4δ2 ratio in subjects with TB is unchanged compared to controls. As discussed in chapter 5 the cross-sectional data provided in this study provides only a 'snap-shot' of the IL-4/ IL-4δ2 ratio. It is entirely possible, as it is in a mouse model (R Hernado-Pandez, personal communication), that the ratio is different in the crucial early phase of the disease when IL-4 may tip the balance in favour of progression.

Collectively, these data provide circumstantial evidence for the role of IL-4δ2 as a disease modifying antagonist in tuberculosis. However, studies in murine and *in vitro* models, and prospective longitudinal studies in subjects with LTBI and will be required to clarify whether IL-4δ2 is a key molecule in determining TB outcomes. If this is borne out then IL-4δ2 may have utility for the design of immunotherapeutic interventions, as a marker of disease activity and a surrogate marker of protective immunity.

9.4. Th1 responses

Over the last decade a number of studies have shown that there are poor proliferative and TB antigen-specific IFN-γ responses in the peripheral blood of TB patients compared to healthy controls (reviewed in chapter 5). Consequently, it was assumed that robust IFN-γ responses represented a correlate of protection; vaccine candidates have been screened according to this rationale. Remarkably, there are only a handful of studies examining cytokine profiles at the site of disease, the lungs, in pulmonary tuberculosis and even fewer in HIV-TB co-infected patients. The data presented here confirm that, in contrast to whole blood, in the lungs there are high levels of IFN-γ.

Even in HIV+ subjects the problem does not appear to be a lack of IFN- γ producing cells (chapter 8, [530]). Clearly, studies in donors with genetic defects [344] have shown that a certain minimum level of IFN- γ is required for protection. However, the current study supports the notion that something else is required, and it is the nature of the Th1 response that is important (mixed Th1/Th2 or a regulatory response, or a combination) not the quantity. Further evidence supporting this contention is discussed in detail in chapter 5 (section 5.3.4).

9.5. Implications for vaccine design and future directions

Almost all TB vaccine candidates have been screened for their Th1 driving capacity in pathogen free laboratory mice, unlike the human situation where there are pre-existing mixed Th1/Th2 responses. There are exceptions, which down regulate Th2 responses whilst concurrently boosting Th1 responses (*M. leprae* hsp65 DNA vaccine and multiple dose *M. vaccae*, discussed in chapter 7, section 7.3.2 and 7.3.3). More than 200 potential TB vaccine candidates have now been identified but the capacity to test them in humans is restricted due to limited field testing-sites and monetary resources. Therefore, candidates will need to be prioritised based on available scientific data. As such, the existing murine data and additional human data presented here and elsewhere (reviewed in [12]) will facilitate the prioritisation of such candidates for evaluation in clinical trials. In parallel, other antigens will need to be screened for their Th2 inducing capacity by using methods discussed in chapter 7, section 7.3.3 (attempts to initiate these studies have now begun). Once identified these antigens could be excluded from existing vaccine preparations (recombinant live vaccines or fusion proteins), or administered together with adjuvants that can drive Th2 suppressing regulatory cells or alter antigen-specific T-helper responses. Antigens that drive IL-4 δ 2 but not IL-4 may also be important. Even if candidates that down regulate Th2 responses prove ineffective when used by themselves they may prove to be useful as adjuvants. Neutralising antibody to IL-4, reported to be therapeutic in the mouse model [499], is available and requires further clinical study in humans.

Now that therapeutic modulators of mRNA stability are a reality it would be instructive to determine whether post-transcriptional mRNA regulation of IL-4

(chapter 4) or other biological molecules would be beneficial in models of TB. Data are required about IL-4 mRNA stability in T cell-subpopulations (lung and peripheral blood), and whether inhibitors of mRNA stability can attenuate markers of immunopathology in *in vitro* models. Whether the balance between IL-4 and IL-4δ2 is instrumental in determining disease outcomes requires further study in longitudinal human studies and laboratory models. Development of antibodies to distinguish between the variants would facilitate this process. IL-4δ2 also requires further study as a potential marker of disease activity and correlate of protective immunity. To what extent regulatory T cells modulate Th2 profiles in tuberculosis requires further study. Particularly relevant to vaccine design would be to determine how TB antigens interact with DCs to determine Th-cell differentiation and regulation. Because of the close interaction between HIV and tuberculosis many of these considerations can be extended to HIV-TB co-infection and the role of Th2 cytokines in facilitating viral replication in co-infected patients requires further study. Finally, the current study has highlighted that, for meaningful interpretation of immunological data, future studies must use biological samples from the site of disease and peripheral blood.

9.7. Conclusion

The work presented in this thesis led to the development of a reproducible quantitative assay that will facilitate future work related to IL-4 and IL-4δ2. The validation of a suitable reference will facilitate the use of real-time PCR in studies unrelated to tuberculosis. It has been demonstrated that a Th2-like response, prominent in T cells, and driven by TB antigen, is present in pulmonary tuberculosis. This response is associated with disease extent and is modulated by treatment. Furthermore, enhancement of IL-4 mRNA stability, previously undescribed in tuberculosis, may represent an additional mechanism by which *M. tuberculosis* may drive immunopathology. Further studies are required to determine whether IL-4 facilitates systemic HIV progression in co-infected patients. Collectively, these data suggest a role for IL-4 and its antagonist, IL-4δ2, in the pathogenesis of TB and their ratio as a possible marker of disease activity and outcome.

The data presented here cannot definitively prove a causal relationship between IL-4 and tuberculosis susceptibility. Nor can it prove that an ideal vaccine is one that will

drive a Th1 response but simultaneously suppress pre-existing Th2 responses. However, it is hoped that that publications arising out of this work will help to prioritise vaccines, which downregulate Th2 responses, for clinical trial. The next logical step is to identify IL-4 inducing antigenic components, which can be manipulated for vaccine design. Funding and protocols to facilitate this goal are already in progress. I hope that clarification provided by the current work and future initiatives will one day translate into benefit for people who might otherwise succumb to *M. tuberculosis*.

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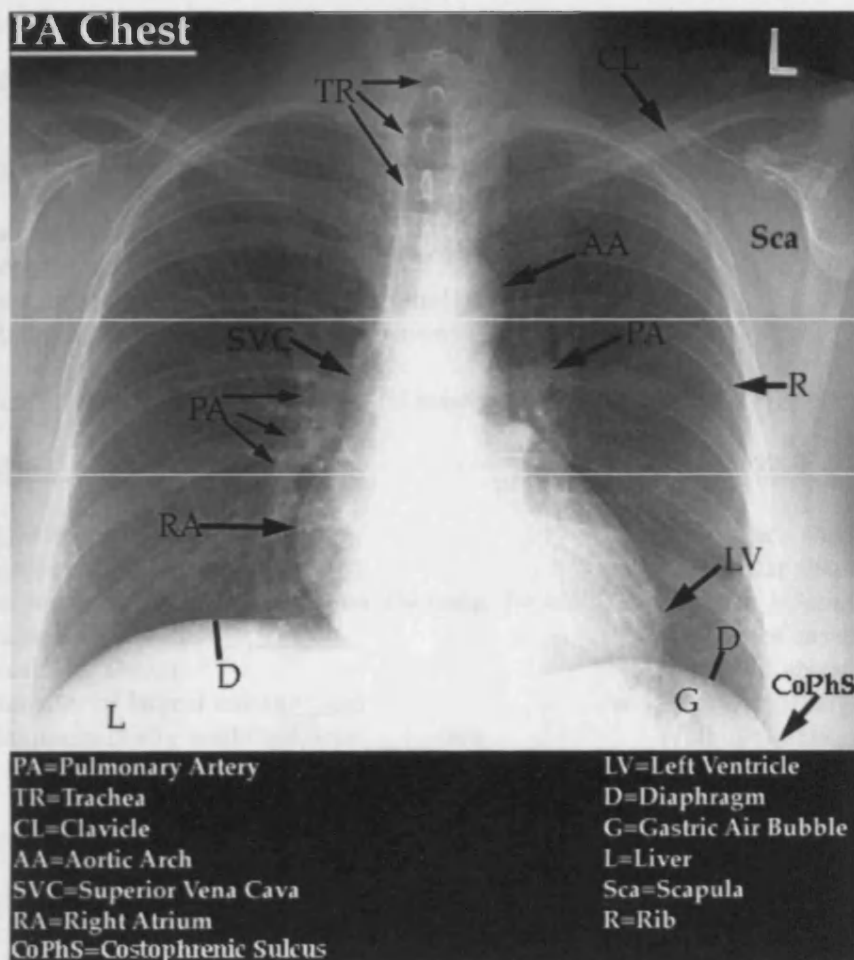
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APPENDIX

Appendix 1. X-ray scoring for pulmonary tuberculosis



Upper zone: between a horizontal line intersecting the 2nd costochondral junctions, and upwards from this line to the lung apices

Middle zone: from the 2nd to the 4th costochondral junction

Lower zone : from the 4th junction downwards

Appendix 1.1. Radiographic scoring of pulmonary tuberculosis.

Scoring system: For the purposes of scoring each lung was divided into 3 zones (shown above). The involvement in each zone was graded (0= none; 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3). In this way 'airspace shadowing' and 'reticular shadowing' was graded for all 6 zones

Cavitation in each zone was graded as present or absent, the number of cavities documented and further details noted (maximum cavity diameter of the cavity and maximum wall thickness). Furthermore, fibrotic lung contraction in each lung was assessed as being present or absent (tracheal deviation, hilar movement, movement of fissures and diaphragmatic tenting). Other features were recorded as being present or

absent (pleural thickening or effusion, pericardial effusion, atelectasis, bronchiectasis and miliary shadowing). In this way a final 'lung remodelling' score (minimum 0 and maximum 100) was generated (an example of the scoring form and method is shown below). Chest radiographs were scored by 2 radiologists blinded to patient details and stage of disease.

Sample X ray scoring form: Dept. of Radiology and Thoracic Medicine, RFH

Patient initials:

Hospital number:

Time in relation to TB treatment (initial or 6 months) :

(Radiologist blinded to this information)

Note: each space must be filled (if none mark with 0 or n/a if not applicable).

Zone 1

% air space shadowing: _____

% reticular shadowing: _____

(air-space and reticular shadows, 0= none, 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3)

Number of cavities: _____

(mark 0 if absent)

Diameter of largest cavity _____cm

Maximum cavity wall thickness _____mm

Zone 4

% air space shadowing: _____

% reticular shadowing: _____

(air-space and reticular shadows, 0= none, 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3)

Number of cavities: _____

(mark 0 if absent)

Diameter of largest cavity _____cm

Wall thickness _____mm

Zone 2

% air space shadowing: _____

% reticular shadowing: _____

(air-space and reticular shadows, 0= none, 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3)

Number of cavities: _____

(mark 0 if absent)

Diameter of largest cavity _____cm

Maximum cavity wall thickness _____mm

Zone 5

% air space shadowing: _____

% reticular shadowing: _____

(air-space and reticular shadows, 0= none, 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3)

Number of cavities: _____

(mark 0 if absent)

Diameter of largest cavity _____cm

Wall thickness _____mm

Zone 3

% air space shadowing: _____

% reticular shadowing: _____

(air-space and reticular shadows, 0= none, 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3)

Number of cavities: _____

(mark 0 if absent)

Diameter of largest cavity _____cm

Maximum cavity wall thickness _____mm

Zone 6

% air space shadowing: _____

% reticular shadowing: _____

(air-space and reticular shadows, 0= none, 1= <1/3; 2= between 1/3 and 2/3 and 3= >2/3)

Number of cavities: _____

(mark 0 if absent)

Diameter of largest cavity _____cm

Wall thickness _____mm

1. Evidence of fibrotic distortion (tracheal deviation, hilar displacement, fissure movement, diaphragmatic tenting or other)

Right lung: Yes _____ No _____

Left lung: Yes _____ No _____

Feature present _____

Feature present _____

2. Pleural reaction (thickening and/or effusion; can only conclude from 2nd film)

Right lung: Yes _____ No _____

Left lung: Yes _____ No _____

Feature present _____

Feature present _____

Extent _____

Extent _____

(blunting of CP angle, < 50% of pleural margin, > 50% of pleural margin)

3. Other findings or comments (bronchiectasis, atelectasis, lymph nodes, pericardial effusion, calcification, miliary pattern, nodules)

SCORING:

- a. To score extent of air space shadowing: add all 6 zones to give a raw score of between 0 and 18.
- b. To score extent of reticular shadowing: add all 6 zones to give a raw score of between 0 and 18.
- c. Is cavitation absent or present: _____
Total number of cavities: _____
Maximum diameter (cm): _____
Maximum wall thickness (mm): _____
[No cavities then score 0,
one to three cavities on CXR and max diameter < 3cm and max wall thickness < 3mm then score 9,
more than three cavities or max diameter of any cavity > 3cm or max wall thickness of any cavity \geq 3mm, then score 18].
(Maximum score = 18, Minimum = 0).
- d. Evidence of lung contraction absent, present 1 side or present bilaterally:
Absent score 0, present on any side score 18

Total extent of disease score: $[(a+b+c+d) \times 100] / 72$
= _____ (lung remodelling index out of 100)

Appendix 2: FACS buffer

The following constituents were dissolved in 490 ml PBS (pH 7.4):

5 ml 1% FCS

5 ml of 0.02% sodium azide

Appendix 3: Phosphate-buffered saline (PBS)

The following constituents were dissolved in 1000 ml of distilled H₂O (pH 7.4):

8 g NaCl

1.44 g Na₂HPO₄

0.24 g KH₂PO₄

0.2 g KCl

Appendix 4: Agarose gel electrophoresis

Agarose gels (3%) were run (120 V, 300 mA for 30 min) in 50 ml tanks (5 mls 10x buffer TBE, 45 mls H₂O, 1.5g agarose (Gibco) and 1µl ethidium bromide), with running buffer (1x TBE with 15 µl ethidium bromide/litre) and 2 µl molecular weight marker (100 and 20 bp ladders, Sigma, UK). Buffer TBE (tris-borate-EDTA) was made to a final volume of 1 litre using de-ionised water, 108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA (pH 8.0). Gels were scanned using a fluorescence image analyser (FLA-2000, Fuji Photo Film).